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Role of amyloid precursor protein in neural stem/progenitor cell proliferation and differentiation

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The research associated with this thesis abides by the international and Australian codes on animal experimentation, and the rulings of the Animal Ethics Committee of the University of Tasmania.

Yanling Hu

Statement of Co-Authorship

Yanling Hu has incorporated a version of the published open access paper Hu et al into Chapter 3 of the thesis.

This work was co-authored with Dr. Amos Hung, Dr. Hao Cui, Dr. Edgar Dawkins, Dr. Marta Bólós, Dr. Lisa Foa, Dr. Kaylene Young, and Prof David Small. Some of the research, data analysis was contributed by Dr. Amos Hung and Dr. Marta Bolós. The majority of the research, analysis, figures were generated and prepared by Yanling Hu herself.

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The contribution of authors to the publication is as follows:

Yanling Hu was the primary author, conducted data analysis, was involved in laboratory experiments and study design and manuscript preparation; Dr. Amos Hung designed and performed part of the neural stem progenitor cell assay; Dr. Hao Cui and Dr. Edgar Dawkins provided technical advice for immunocytochemistry and immunoblotting; Dr. Marta Bolós performed real time PCR; Dr. Lisa Foa contributed to manuscript preparation and study advice; Dr. Kaylene Young provided technical support and experimental design suggestions. Prof. David Small assisted with the scientific direction and provided input into research design. All authors contributed to the final manuscript preparation.

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Table of Contents

Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative condition that commonly affects people over the age of 65. There are currently no effective treatments which prevent or delay the progression of AD. The disease is characterized by two major pathological hallmarks in the brain, extracellular amyloid plaques and intracellular neurofibrillary tangles.

The β-amyloid protein (Aβ) is the major component of the amyloid plaques and is considered to play a central role in AD pathogenesis. Aβ is produced by proteolytic processing of the amyloid precursor protein (APP). However, the normal function of APP remains unclear despite numerous studies. Understanding the biological function of APP may improve our understanding into the molecular basis of AD pathology.

APP expression has been reported to increase in neurons during embryogenesis. In addition, enhanced neurogenesis has been observed in the brains of AD patients and in transgenic mice which overexpress APP. However, other studies have reported impaired neurogenesis in APP transgenic mice. Thus, the studies presented here were aimed at investigating the role of APP in neural stem/progenitor cell (NSPC) proliferation and differentiation.

To test the effect of APP on NSPC proliferation, NSPCs derived from human APP overexpressing transgenic (Tg2576) mice and APP knockout (APP KO) mice and their corresponding background strain wild type (WT) mice were cultured in a proliferation medium containing growth factors and NSPC growth was measured. The study found than Tg2576 NSPCs proliferated more rapidly than NSPCs from WT

control mice. In contrast, NSPCs derived from APP KO mice proliferated less readily than the corresponding background strain mice.

The secreted fragments of APP, Aβ and sAPPα, have been reported to affect neurogenesis. The molecular basis of the effect is uncertain. To examine whether sAPPα or two major isoforms of Aβ, $A\beta_{40}$ and $A\beta_{42}$, were responsible for APP - induced NSPC proliferation, the WT NSPCs were grown in proliferation medium containing sAPPα, $A\beta_{40}$ or $A\beta_{42}$ and the cell proliferation was measured by examining cell viability. No significant difference in cell viability was found between the non – treatment groups and the groups treated with sAPPα, $A\beta_{40}$ or $A\beta_{42}$. Besides, immunodepletion of secreted fragments of APP (sAPPα, Aβ) from Tg2576 conditioned medium did not lower NSPC proliferation, indicating that neither sAPPα nor Aβ contributed to the proliferation effect.

To examine whether other secreted factors might be involved in the proliferation effect, the ability of cell conditioned medium to stimulate proliferation was tested. NSPC conditioned medium from Tg2576 cultures was found to increase proliferation while conditioned medium from APP KO cultures was found to have a lower effect on proliferation. The effect on proliferation was found to be due to a secreted factor, cystatin C (CysC), which has previously been reported to promote NSPC proliferation. Immunodepletion of CysC from the Tg2576 conditioned medium removed the stimulatory effect of APP on NSPC proliferation. mRNA levels of CysC in APP KO cells were lower than the wild-type control cells. Therefore, CysC is likely to be a major mediator of APP-associated NSPC proliferation.

CysC is well characterized as a cysteine protease inhibitor. Proteases have been reported to play a role on cell proliferation, thus regulation of proteases activities may affect NSPC proliferation. To examine whether the effect of CysC on NSPC proliferation was due to an effect on one or more cysteine proteases, NSPCs were treated with the broad spectrum protease inhibitor, chymostatin, the specific cysteine protease inhibitor, E-64, the aspartic protease inhibitor, pepstatin A and the serine and cysteine protease inhibitor, antipain, after which cell proliferation was examined. The cysteine protease inhibitor, E-64 and the serine/cysteine protease inhibitor, antipain, both of which are cell membrane impermeable were found to stimulate NSPC proliferation at low concentrations. However, no extracellular cysteine proteases were identified bound to CysC in the conditioned medium by the CysC affinity chromatography. Thus more studies may be required in the future to identify the specific target of CysC.

To test the effect of APP on NSPC differentiation, NSPCs prepared from Tg2576, and APP KO and corresponding background strain wild type mice were cultured in a differentiation medium lacking of growth factors. Cultures were immunostained for markers of neurons (βIII tubulin), astrocytes (GFAP) and oligodendrocyte progenitors (NG2) various days after incubation to assess cell differentiation. APP overexpressing (Tg2576) NSPC cultures displayed more βIII tubulin$^+$ and GFAP$^+$ cells than the wild type cultures, while APP KO NSPC cultures had fewer βIII tubulin$^+$ and GFAP$^+$ cells compared to the corresponding wild type cultures. No significant difference was found in NG2 expression.

Previously published studies on the effect of sAPPα, $A\beta_{40}$ and $A\beta_{42}$ on NSPC differentiation have yielded conflicting results. Therefore, one of the aims of this study was to examine whether CysC, sAPPα, $A\beta_{40}$ and $A\beta_{42}$ influence NSPC differentiation. The APP KO NSPCs were grown in differentiation medium containing CysC, sAPPα, $A\beta_{40}$ and $A\beta_{42}$, and the cells were fixed and immunostained for markers of neurons (βIII tubulin), astrocytes (GFAP) and oligodendrocyte progenitors (NG2). No difference in the expression of the neuronal and glial markers between the control group and groups treated with CysC, sAPPα, $A\beta_{40}$ and $A\beta_{42}$ was observed. It was concluded that neither CysC nor sAPPα, Aβ influence NSPC differentiation.

In summary, the study found that APP can promote NSPC proliferation *in vitro*, and the effect is mediated by secreted CysC. However, the molecular basis of CysC action requires further studies. In addition, APP was also found to play a role on NSPC differentiation *in vitro*. Adult neurogenesis has been reported to play important roles in learning, memory and mood regulation. Therefore, therapeutic strategies focusing on anti – Aβ production by alteration APP processing should be considered carefully. Besides, APP - directed neuronal differentiation of NSPCs could perhaps contribute to stem cell therapy for neurodegenerative disease treatments including AD.

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List of Abbreviations

AChE	Acetylcholinesteras
AD	Alzheimer's disease
ADAM	A disintergrin and metalloproteinase
AICD	APP intracellular domain
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3 hydroxy-5-methyl-isoxazolepropionic acid
AMPAR	AMPA receptor
ANOVA	Analysis of variance
APLP1	APP – like protein 1
APLP2	APP – like protein 2
apoE4	Apolipoprotein E4
APH-1	Anterior pharynx-defective 1
APP	Amyloid precursor protein
APP KO	APP knockout
APP dKO	APP/APLP2 knockout
APP tKO	APP/APLP1/APLP2 triple knockout
APPsw	APP carrying the Swedish mutation (K670N/K671L)
BACE	β – site APP cleaving enzyme
BACE KO	BACE knockout
BBB	Blood - brain barrier
BDNF	Brain derived neurotrophic factor
BIN1	Bridging integrator 1
BSA	Bovine serum albumin
bHLH	basic helix -loop - helix
BMP	Bone morphogenetic protein
CAA	Cerebral amyloid angiopathy
CalB	Calbindin
CALHM1	Calcium homeostasis modulator
CCR	Chemokine receptors
CNS	Central nervous system
CNBr	Cyanogen bromide

CSF	Cerebrospinal fluid
CysC	Cystatin C
CTS3	Gene coding CysC
CuBD	Copper – binding domain
DAPI	4′,6-diamidino-2-phenylindole
DCX	Doublecortin
DG-SGZ	Subgranular zone of dentate gyrus
ECM	Extracellular matrix
EdU	5-ethynyl-2'-deoxyuridine
EGC	Embryonic germ cells
EGF	Epidermal growth factor
EGFR	EGF receptor
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EOAD	Early - onset AD
ESC	Embryonic stem cell
FAD	Familial AD
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGFR	FGF receptor
FTD	Frontotemporal dementia
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Granule cells
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acid protein
GFLD	Growth factor like domain
GLAST	Glutamate aspartate transporter
GSK-3β	Glycogen synthase kinase-3β
GWAS	Genome-wide association study
HCCAA	Hereditary cystatin C amyloid angiopathy
HBD	Heparin binding domain
HD	Huntington's disease
ICC	Immunocytochemistry
IFγ	Interferon gamma

IGF	Insulin growth like factor
iPSC	Induced pluripotent stem cells
KPI	Kunitz protease inhibitor domain
LDL	Low-density lipoprotein
LDLR	Low density lipoprotein receptor
LRP	Lipoprotein protein receptor
LTD	Long term depression
LTP	Long term potentiation
LOAD	Late - onset AD
MAP	Microtubule - associated protein
MAPK	Mitogen activated protein kinase
MCI	Mild cognitive impairment
mGluR7	Metabotropic glutamate receptor 7
MSC	Mesenchymal stem cell
NCT	Nicastrin
NGF	Nerve growth factor
Ngn2	Neurogenin2
NICD	Notch intracellular domain
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
NSC	Neural stem cell
NSPC	Neural stem/progenitor cell
NFT	Neurofibrillary tangles
NMJ	Neuromuscular junction synapses
OB	Olfactory bulb
OPC	Oligodendrocyte progenitor
PBS	Phosphate-buffer saline
PD	Parkinson’s disease
PEN	Presenilin enhancer
PDGF-APPsw,Ind	APP carrying the Swedish (KM670N/671L) and Indiana (V717F) mutation
PGC	Periglomerular cell
PID	Phosphototyrosine interacting domains

Pin1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting1
PS	Presenilin
PSA-NCAM	Polysialylated neural cell adhesion molecule
PTB	Phosphotyrosine binding domain
PVDF	Polyvinylidene difluoride
RMS	Rostral migratory stream
sAD	Sporadic AD
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SGZ	Subgranular zone
Shh	Sonic hedgehog
siRNA	Small interfering RNA
SNAP25	Synaptosomal-associated protein 25
TACE	Tumour necrosis factor α converting enzyme
TBS	Tris buffered saline
TBS-T	Tris buffered saline Tween -20
TFs	Transcriptional factors
TH	Tyrosine hydroxylase
Tip60	Tat-interactive protein 60
TLX	A member of the nuclear receptor family of intracellular transcription factors
TNFα	Tumour necrosis factor α
TREM2	Triggering receptor expressed on myeloid cells 2
Trk	High affinity nerve growth factor receptor
UR	Unstructured region
VEGF	Vascular endothelial growth factor
V-SVZ	Ventricular – subventricular zone
Wnt	Wingless
WT	Wild type

Chapter 1 Introduction

1.1 Alzheimer's disease (AD)

The study presented in this thesis was focused on elucidating one of the functions of amyloid precursor protein (APP) of Alzheimer's disease (AD). Because of the importance of APP for AD, this chapter first reviews AD and the central role of APP in AD pathogenesis.

AD is a neurodegenerative disease and the leading cause of dementia in the elderly. Approximately 2% of the population between the ages of 65 and 69 years suffer from AD. However, this figure dramatically increases to 25 to 30% between the ages of 80 to 85 (Vandenberghe & Tournoy 2005). The pathology of AD was first described in a 51 year old female patient by the German psychiatrist, Alois Alzheimer (Alzheimer 1907). There is still no cure, or even an effective treatment, to delay the progression of AD.

1.1.1 Clinical features of AD

During the progression of AD, brain cells are damaged or become dysfunctional, which gradually leading to the loss of memory, thinking, cognition and other physiological functions regulated by the brain, including motor activity (Tarawneh & Holtzman 2012). The combination of memory decline, language disturbance, motor activity and cognitive impairment clinically describes dementia (Tarawneh & Holtzman 2012). However, a diagnosis of AD only can be confirmed by post mortem pathological analysis. Therefore, ante-mortem diagnosis of AD depends on the analysis of clinical symptoms (Tarawneh & Holtzman 2012). At an early stage of dementia, diagnosis of AD is difficult because some memory decline is normally associated with aging. At an early stage of AD, patients exhibit mild cognitive

impairment (MCI) and a deficiency in short-term memory and information storage (Bayley et al 2000, McCormick et al 1994, Morris et al 2001). Besides aggressive behavior, the majority of MCI patients show mild psychological symptoms including anxiety, depression, delusions, hallucinations and mood changes (Gauthier et al 1997, Mega et al 1996, Weiner et al 2005). Moreover, patients at this stage have problems with calculation, insight and judgment, but neurological function usually is normal (Tarawneh & Holtzman 2012). When AD develops to a moderate stage, intensive or permanent supervision may be required because patients experience cognitive behavioral problems, somatic symptoms, impairment of recent memories, sleep disturbances and loss of emotional control (Forstl & Kurz 1999, Rebok et al 1991). In the final (severe) stage of AD, patients potentially lose the ability to control bodily functions and have profound circadian rhythm disturbances (Forstl & Kurz 1999, Volicer et al 2001). Therefore, full-time nursing care is required (Herrmann & Gauthier 2008). Clinical diagnosis of dementia mostly occurs during the mild to moderate stages of the disease and death usually occurs 5-8 years after diagnosis (Forstl & Kurz 1999). Patients with dementia most frequently die of bronchopneumonia, cerebrovascular or cardiovascular disease (Brunnström & Englund 2009).

1.1.2 Impact and prevalence of AD

AD accounts for 50%-70% of all cases of dementia. Patients with a moderate to severe stage of AD rely heavily on caregivers, and at a severe stage of the disease usually require institutionalisation. Therefore, the total cost of dementia is a major challenge for the healthcare system. In Australia in 2009, there are more than 321,000 people living with dementia, and the cost of the dementia and aged care sector is at

least USD $4.9 billion annually, or around 1% of GDP (Access Economics 2009, Australian Institute of Health and Welfare 2012). In the United State of America, 5.2 million people have AD including 200,000 individuals (Hebert et al 2013) under 65 who have early-onset AD (Alzheimer's Association 2006). The total cost of care for people living with AD and other dementias in USA is estimated to be USD $214 billon (Alzheimer's Association 2014). Furthermore, more than 36 million people worldwide live with dementia and the total estimated worldwide cost is estimated to be around 604 billion (Alzheimer's Disease International 2012). However, the number of people living with dementia in the population around the worldwide is predicted to be 115 million by 2050 (Alzheimer's Disease International 2012), with spending on dementia treatment and care estimated to outnumber that of any other health condition by 2050 (Access Economics 2009). Therefore, it is crucial to find therapies that can block or delay the progression of AD. Development of a therapy for AD would not only relieve the enormous burden on the health care system, but more importantly, it would improve the quality of life for individuals with AD and their families.

1.1.3 Pathology of AD

The development of lesions in the vulnerable sites of the brain is thought to be the central pathological processes in many neurological diseases, including AD (Braak et al 2006), Thus, investigation into the role that pathological lesions play in disease pathogenesis is important. A number of pathological lesions are typically found in the post-mortem AD brain. Pathological lesions such as extracellular plaques, intracellular neurofibrillary tangles and adipose inclusions were originally identified using silver-staining techniques by Alois Alzheimer (Alzheimer 1907, Alzheimer et al 1995). Neuron and synapse loss (Terry et al 1991b, Whitehouse et al 1982), and

amyloid deposits in brain blood vessels (cerebral amyloid angiopathy; (Revesz et al 2003)) were identified in later histopathological investigations of AD brain sections.

1.1.3.1 Neurofibrillary tangles

One of the hallmarks of AD pathology is the intracellular accumulation of neurofibrillary tangles (NFTs) in neurons. The NFTs develop in the cell body and can spread into the dendrites. Once nerve cells have been destroyed, the NFTs convert to an extraneuronal structure which can eventually be taken up and degraded by astrocytes (Braak & Braak 1991b). In the brain of AD patients, NFTs generally exhibit a common and highly predictable pattern of distribution with only minor inter-individual variations (Braak & Braak 1991b). NFTs develop in a temporal progression that correlates with the clinical symptoms of AD (Bierer et al 1995). The progression of NFT pathology has been characterized in six stages (Braak & Braak 1991b). In stages I and II, NFTs accumulate in the transentorhinal region and in the CA1 region of the hippocampus (transentorhinal stage). NFTs develop severely in transentorhinal and entorhinal regions but moderately in the hippocampus during stage III and IV (limbic stage). Finally, numerous NFT aggregates can be seen in the hippocampus and isocortex in stage V and VI. The isocortex is severely affected in these stages. Therefore, stages V and VI can be interpreted as an isocortex stage (Braak & Braak 1991b). Stage I and II are a nonclinical pathology, and can be found in individuals as young as 20. The limbic stage (Stage III and IV) is associated with emerging AD pathology, and can be found in 10% of adults of aged 50 or over, and 50% of adults 80 years old or more (Braak et al 2011). The isocortex stage (stage V and VI) is regarded as fully developed AD, which is present in 10% of adults aged 80, and 20% of adults aged 90 (Braak & Braak 1995).

NFTs are composed of paired helical filaments that consist of an abnormally hyperphosphorylated form of the microtubule-associated protein (MAP) tau (Grundke-Iqbal et al 1986, Kosik et al 1986, Wood et al 1986). Tau is a type II MAP, that modulates microtubule stability inside axons and facilitates the assembly of microtubule bundles within the nerve cell (Mandelkow & Mandelkow 1995). Normally, tau binds to the outer ridge of the neuroprotofilaments and works as an anchor to stabilise microtubule bundles or regulates the dynamics of microtubule assembly (Al-Bassam et al 2002). When tau is hyperphosphorylated, it detaches from protofilaments and loses the ability to promote assembly of, and maintain the structure of, microtubules (Grundke-Iqbal et al 1986). In addition, the cytosolic abnormally hyperphosphorylated tau can also cause normal tau and other MAPs to dissociate from microtubules and to block microtubule assembly (Iqbal et al 2008). Moreover, the level of abnormally hyperphosphorylated tau in the AD brain is significantly higher than the normal brain (Khatoon et al 1992). Therefore, hyper-phosphorylated tau may lead to disruption of the microtubule assembly and may cause affected neurons to undergo a slow but progressive neurodegeneration (Iqbal et al 1994). However, the mechanism causing the development of abnormal tau aggregates needs clarification.

1.1.3.2 Amyloid plaques

Amyloid plaques are focal lesions that are another hallmark of AD (Braak & Braak 1991b). Generally, extracellular amyloid plaques are present in all six layers of the isocortex, but are most abundant in layer V. Unlike NFTs, amyloid plaques develop in an unpredictable pattern of distribution pattern with predominant individual variation. The progression of amyloid plaques can be roughly classified into three stages. In

stage A, a low density of amyloid deposits is found in the isocortex, particularly in the basal portions of the frontal, temporal and occipital lobe but deposits are almost absent from the hippocampus (Braak & Braak 1991b). However, a few amyloid plaques can be found in the junction between the hippocampus and isocortex. In stage B, amyloid plaques are encountered in nearly all the isocortical association areas and are also present in the white matter underlying the cortex. The hippocampus is mildly affected, but the primary sensory region and the primary motor area remain devoid of amyloid plaques at this stage. In stage C, all isocortex areas are loaded with amyloid in a laminar distribution. Some regions outside the cerebral cortex including the striatum, thalamus, hypothalamus, subthalamic nucleus and red nucleus also exhibit deposition of amyloid, but the hippocampus still harbours comparatively fewer deposits (Braak & Braak 1991b). Clinical pathological studies indicate that amyloid burden does not correlate with the severity and duration of dementia. Furthermore, the size of the plaques does not increase with the progression of AD (Arriagada et al 1992, Bierer et al 1995, Giannakopoulos et al 2003, Gomez-Isla et al 1997, Hyman et al 1993). However, preliminary data from longitudinal amyloid PET imaging studies of living patients suggest that the total amount of amyloid deposits in the cortical mantle increases during the clinical course of AD, and that amyloid plaques spread according to the stages described above (Jack et al 2009).

Amyloid plaques are characterized by accumulation and deposition of amyloid beta peptides (Aβ) that are produced from the normal proteolysis of the β-amyloid precursor protein (APP) (Kang et al 1987). Aβ can aggregate into amyloid fibrils that subsequently deposit to form amyloid plaques. Although Aβ is the major ingredient of amyloid plaques (Glenner & Wong 1984, Masters et al 1985, Selkoe et al 1986), a

number of other components in the plaque can also be identified including apoE, clusterin and cystatin C (CysC) (DeMattos et al 2001, May et al 1990, Sanan et al 1994, Yamada et al 1989). Current methods to classify different types of plaques in AD brains are generally based on morphological characteristics (D'Andrea & Nagele 2002). Amyloid plaques can be distinguished into two types depending on the β–pleated sheet conformation of the insoluble fibrous protein aggregates (β-amyloid aggregate) that can be specifically stained with Congo Red or Thioflavin-S (Sipe et al 2010). Plaques that are Thioflavin positive are dense core plaques, while those that accumulate immunoreactive Aβ, but which are Congo Red or Thioflavin negative, are diffuse plaques (Tagliavini et al 1989, Yamaguchi et al 1988). Plaque types correlate with disease. The Thioflavin-S positive dense core plaques consist of a central mass of amyloid filaments that radially project to the periphery where they interact with neuronal, astrocytic and microglial processes (Serrano-Pozo et al 2011). Several studies indicate that dense core plaques closely correlate with dystrophic neurites (neuronal processes), neuron loss, synaptic loss, and astrocytic and microglial activation (Itagaki et al 1989, Knowles et al 1999, Pike et al 1995, Urbanc et al 2002, Vehmas et al 2003). Dense core plaques, especially those associated with dystrophic neurites, are always present in the brains of AD patients (Sheng et al 1998, Urbanc et al 2002), whereas amorphous diffuse plaques can be found commonly in the brains of elderly individuals with intact cognition (Morris et al 1996, Yamaguchi et al 1989). The diffuse plaques are not typically related to dystrophic neurites and synapse loss (Masliah et al 1990, Morris et al 1996). Plaque-associated neuritic dystrophy provides evidence that amyloid plaques contribute to neurodegeneration in AD. Moreover, the phosphorylation of tau can be induced by the major component of the amyloid plaque – Aβ (Chabrier et al 2012, Gotz et al 2001, Jin et al 2011, Tokutake et al 2012).

Therefore, tau aggregates are most probably a downstream pathology mediated by Aβ.

1.1.3.3 Cerebral amyloid angiopathy (CAA)

Amyloid deposited in the wall of cerebral and leptomeningeal vessels is called cerebral amyloid angiopathy (CAA). CAA pathology commonly appears in individuals with dementia, and can be observed in approximately 80% of AD patients (Jellinger 2002). Furthermore, post-mortem longitudinal studies reveal that CAA may play a role in cognitive decline in AD (Arvanitakis et al 2011, Greenberg et al 2004, Pfeifer et al 2002). Cerebral amyloid deposits can often be found in leptomeningeal and cortical arteries, and arterioles, but less frequently is present in capillaries and veins of the central nervous system (CNS) (Pezzini et al 2009). After deposition in the outer membrane, and between smooth muscle cells of the blood vessel, CAA can weaken and destroy blood vessels leading to intracerebral haemorrhage and cerebral infarction (Pezzini et al 2009). The major ingredient of the cerebral amyloid deposits in AD-related CAA is Aβ (Herzig et al 2004). However, other proteins (CysC, prion protein, transthyretin, and gelsolin) can form deposits in the walls of cerebral blood vessels to form CAA in other diseases (Pezzini et al 2009). For instance, in hereditary CysC amyloid angiopathy (HCCAA), CysC accumulation in the vascular media and adventitia causes progressive loss of smooth muscle cells leading to cerebral haemorrhage (Wang et al 1997).

Studies reveal that vascular lesions may contribute to CAA pathology of AD (Gorelick et al 2011, Thal et al 2003). However, based on autopsy samples based from the Honolulu Asia Aging Study, it is suggested that the CAA burden in AD is

independent of vascular lesions (Launer et al 2008, Pfeifer et al 2002). Therefore, vascular disease may make little contributions to the formation of CAA in AD (Launer et al 2008). Formation of CAA pathology in AD is probably more closely associated with amyloid plaque deposition.

1.1.3.4 Synapse loss

Synaptic transmission is essential for cognition. Therefore, synapse loss is thought to be the major correlate of cognitive impairment in dementia including AD (Hamos et al 1989, Terry et al 1991b). Loss of synaptic contacts in both the neocortex and hippocampus is one of the pathological features of AD, and has been demonstrated to be an early event in AD before neuron loss and tangle formation (Masliah 1995, Scheff et al 2006). Synapse loss occurs predominantly in areas surrounding senile plaques, suggesting that amyloid plaques may contain a reservoir of Aβ that causes synaptic toxicity (Moolman et al 2004, Tsai et al 2004).

1.2 β– Amyloid (Aβ)

Extracellular amyloid plaques are primarily composed of Aβ. The hypothesis that Aβ is the main cause of dementia in AD is central to the majority of research in both academia and industry, although the direct relationship between Aβ, memory loss and cognition impairment in dementia has not been fully elucidated. However, considerable evidence from studies using Aβ overproducing mice supports the view that overproduction of Aβ leads to amyloid plaque lesions that are related to AD (Citron et al 1992, Citron et al 1997, Selkoe 1993). Furthermore, various studies indicate that various forms of Aβ aggregates are harmful to neuronal and synaptic

function (Jin et al 2011, Kullmann & Lamsa 2007, Lefterov et al 2009, Shankar et al 2008).

1.2.1 Amyloid precursor protein processing and Aβ production

Aβ is derived by proteolytic processing of amyloid precursor protein (APP) (Sisodia et al 1990). APP is a transmembrane protein, and the Aβ sequence extends from the ectodomain of APP into the membrane spanning-domain (Kang et al 1987). During proteolytic processing, APP can be cleaved by several proteases called secretases. APP is mainly processed by an α-secretase cleavage pathway that is non-amyloidogenic pathway (Sisodia et al 1990) (Fig. 1.1). In this pathway, APP is processed consecutively, at first by α-secretase within the Aß sequence. This releases a 100 kDa extracellular soluble form of APP named sAPPα, leaving an 83 amino acid residue fragment containing the transmembrane and intracellular C-terminal region of APP (C83) (Esch et al 1990, Sisodia 1992). C83 spans the plasma membrane and projects into the intracellular space. C83 can be further cleaved by γ–secretase to produce a small fragment of APP (P3) and the intracellular domain of APP (AICD). In the β-secretase pathway (amyloidogenic pathway), APP is initially cleaved by β-secretase to liberate a N-terminal ectodomain (sAPPß) into the extracellular space. The remaining 99 amino acid residue C-terminal fragment (C99) is then further cleaved by γ–secretase to liberate Aß and the APP intracellular domain AICD. The Aβ can be cut by γ–secretase into a peptide ranging from 37 to 42 amino acid residues in length (Qi-Takahara et al 2005). Different variants of Aβ peptide can be detected as

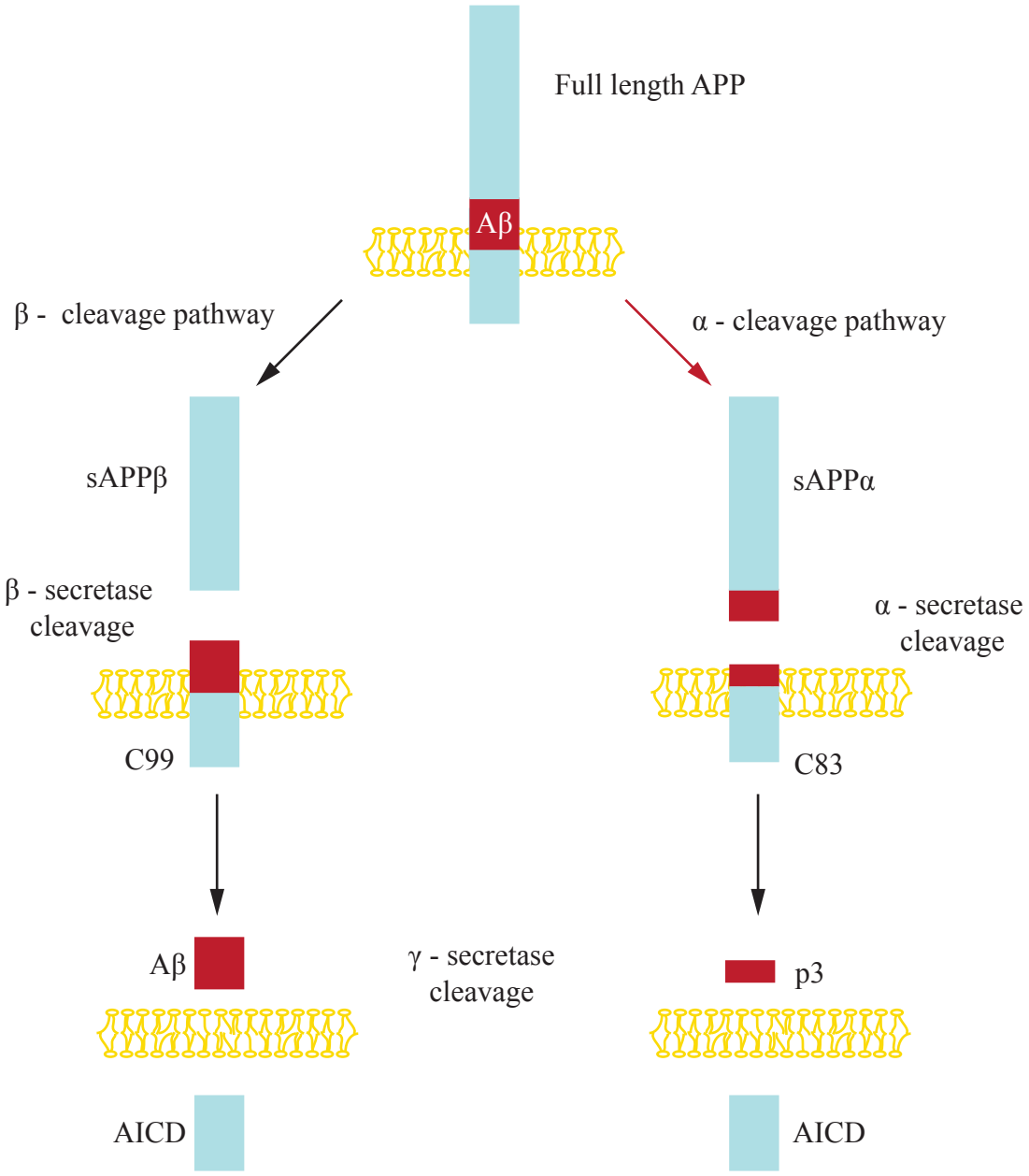

Figure 1.1 Diagram showing the β-secretase cleavage and α-secretase cleavage pathways of APP proteolytic processing. In the β-secretase pathway, APP is cleaved by BACE1 to generate a secreted ectodomain, sAPPβ, and the C - terminal membrane spanning domain, named C99. C99 can be futher cleaved by γ-secretase to produce an intracellular domain fragment (AICD) and the secreted Aβ. The α-secretase pathway is a major processing pathway of APP. In this pathway, APP is cleaved by α-secretase to release a soluble extracellular domain fragment, sAPPα. The C-terminal domain remaining in the membrane, termed C83, is further cleaved by γ-secretase to generate a secreted fragment, p3, and the intracellualr domain (AICD).

a soluble component of plasma cerebrospinal fluid and other tissues, even in normal physiological conditions (Iwatsubo et al 1994, Seubert et al 1992). However, Aβ40 and Aβ42 are the most frequent variants that can be found in amyloid plaques (Iwatsubo et al 1994), and Aβ42 is more common in CAA than Aβ40 (Roher et al 1993). Because of the two extra amino acids, Aβ42 is more prone to aggregate and form fibrils, which are suggested to be more toxic than those formed by Aβ40 (Dahlgren et al 2002, Jarrett et al 1993). Moreover, an increased amount of Aβ42, or an enhanced ratio of Aβ42/ Aβ40, correlates with hereditary forms of AD (Golde et al 2000, Scheuner et al 1996), whereas a decrease in Aβ42 concentration is associated with a lower risk of AD (Weggen et al 2001).

1.2.2 Aggregation of Aβ

Aβ contains a hydrophobic C-terminal region that contributes significantly to β-sheet conformation. The region in the N terminal domain is important for α-helical or β-strand structural switching (Soto et al 1994, Zagorski & Barrow 1992). Aβ predominately forms an α-helical conformation in organic solvents, while in aqueous solution it is more prone to be present in a β-sheet configuration that is closely associated with Aβ fibrillogenesis (Lansbury 1999). However, the transition of Aβ structure is highly dependent on pH and hydrophobicity, which may influence its aggregation and amyloid deposition in the AD brain (Soto et al 1994, Wood et al 1996b).

Aβ containing 39 to 40 amino acid residues is kinetically soluble for hours to days whereas the peptide containing 42 to 43 amino acid residues aggregates immediately (Jarrett et al 1993). In general, multiple Aβ aggregates can be found in amyloid

plaques in the AD brain: fibrils, dimers, monomers, tetramers and the more soluble oligomeric forms of Aβ (Shankar et al 2009). The mechanism of Aβ aggregation to form Aβ deposits and amyloid plaques is still unclear. However, *in vitro* studies on synthetic Aβ proteins suggest several models. Amyloid fibrils are possibly formed through a nucleation-dependent process in which Aβ aggregation requires monomeric Aβ to convert slowly in a nucleation phase to form Aβ oligomers. The addition of Aβ monomers to these oligomers, which are often referred to as 'seed', results in fibril elongation to form longer Aβ aggregates. The reaction eventually reaches a dynamic equilibrium between Aβ aggregates and Aβ monomers. A nucleation–dependent mechanism is identified by a high concentration dependence on 'seed' (nucleus) formation, which leads to Aβ aggregation (Jarrett et al 1993). Esler and his colleagues (Esler et al 2000) reported that Aβ aggregation is propagated by a template dependent 'dock-lock' mechanism that is mediated by two distinct kinetic processes. In the 'dock' phase, Aβ binds to the pre-existing amyloid template in a reversible manner. However, Aβ can, in a time-dependent transition, interact with the template in an irreversible manner. In this 'lock' phase, the deposited 'dock' Aβ interacts irreversibly with the template and is now referred to as 'lock' Aβ. The locked Aβ may then become a new template to which 'dock' Aβ may bind (Esler et al 2000). This provides a template driven mechanism for a transition from Aβ peptides to Aβ aggregates. However, recent structural analysis suggests possible mechanisms of Aβ aggregation. These mechanisms have been recently reviewed (Tycko & Wickner 2013).

1.2.3 Aβ toxicity

Aβ is naturally produced by healthy cells. However, mutations within or around the Aβ sequence lead to elevated Aβ production or changes in Aβ isoforms that have been found to correlate with early-onset AD (Selkoe 1993). Results from many studies suggest that Aβ plays a central role in AD progression, although the underlying mechanism remains to be elucidated (Braak & Braak 1991a, Small et al 2001, Terry et al 1991a).

Aβ induces impaired long-term potentiation (LTP) and thereby alters synaptic transmission. This may contribute to memory and cognitive decline in AD. LTP induces growth of dendritic spines, whereas long-term depression (LTD) facilitates spine shrinkage and synaptic loss (Kullmann & Lamsa 2007). LTP is thought to be closely related to learning and memory (Otto et al 1991), thus excessive accumulation of Aβ may inhibit LTP and lead to memory decline. The memory of a learned behaviour by a normal rat was disrupted after intracerebroventricular injection of the extracted Aβ oligomers (Shankar et al 2008). At high concentrations, soluble Aβ oligomers extracted from the cortex of AD subjects were found to inhibit synaptic LTP and to promote LTD of synaptic transmission (Shankar et al 2008). Aβ-induced LTD can lead to endocytosis of synaptic α-amino-3 hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Hsieh et al 2006, Shankar et al 2008). A reduction of AMPA receptors is necessary and sufficient to induce loss of spines and synaptic (N-methyl-D-aspartate) NMDA responses (Hsieh et al 2006). Recent studies also suggest that Aβ triggers a reduction in the number of cell-surface NMDA receptors (Hsieh et al 2006, Snyder et al 2005). Increased Aβ levels are reported to block uptake of glutamate, which leads to the accumulation of glutamates in the

synaptic cleft (Li et al 2009). Synaptic transmission can requires activation of NMDA receptors. However, persistently elevated glutamate can desensitize NMDA receptors and thereby disrupt synaptic transmission (Li et al 2009). Therefore, pathologically increased levels of Aβ may possibly change NMDA and AMPA receptor signalling pathways, induce LTD, impair LTP, and cause synaptic transmission dysfunction.

Aβ is also suggested to cause functional and structural damage to neurons. Numerous studies show that high levels of synthetic $A\beta_{40}$ and $A\beta_{42}$ can bind to cell membranes and increase membrane permeability, leading to destruction of cellular structures (Arispe et al 1993, Demuro et al 2005, Lin et al 2001). In addition, $A\beta_{42}$ internalization through the endosome/lysosome pathway can lead to lysosomal leakage (Arispe et al 1993, Yang et al 1998). However, natural oligomers of secreted Aβ isolated from culture medium or from brain tissues at nanomolar concentrations have little effect on lipid membrane permeability. This may be explained by the fact that Aβ oligomers induce neuronal dysfunction rather than perturb the membrane (Sanchez-Mejia et al 2008). For example, soluble Aβ oligomers purified from AD cortex are able to induce tau hyperphosphorylation, which further triggers progressive collapse of the microtubule cytoskeleton (Jin et al 2011). Thus, Aβ oligomers contribute to neuronal dysfunction whereas $A\beta_{40}$ or $A\beta_{42}$ probably induce structural destruction.

Calcium homeostasis dysregulation is considered to be another possible mechanism of neuronal dysfunction. Aβ increases the basal levels of calcium and therefore renders neurons more vulnerable to glutamate-induced excitotoxicity eventually leading to neuronal death (Mattson et al 1992, Price et al 1998). Aβ is also reported to

form calcium channels in the cell membrane (Demuro et al 2005). In addition, Aβ has been reported to induce Ca^{2+} influx through binding to NMDA receptors (De Felice et al 2007) or via stimulation of L-type Ca^{2+} channels (Ho et al 2001, Sberna et al 1997). A recent paper indicates that Aβ may impair metabotropic glutamate receptor 7 (mGluR7) on cholinergic neurons, which indirectly affects calcium influx (Gu et al 2014). This implies that disruption of calcium homeostasis may cause selective degeneration of cholinergic neurons, which is one of the pathological features in AD (Gu et al 2014). Moreover, a polymorphism in the calcium homeostasis modulator (CALHM1) 1 gene correlates with an increased risk of AD (Dreses-Werringloer et al 2008). This polymorphism of CALHM1 increases Aβ levels through alteration of Ca^{2+} permeability and cytosolic Ca^{2+} levels (Dreses-Werringloer et al 2008).

However, it is worth mentioning that Aβ-induced neuronal death cannot be thought as the sole cause of cognitive decline in AD (Small et al 2001). In fact, dysfunctional synaptic transmission or the loss of synapses are more correlated with cognitive loss than deposition of Aβ plaques and cell death (Braak & Braak 1991a, Terry et al 1991a).

1.2.4 Possible physiological functions of Aβ

Aβ has been intensively studied for its role in the pathogenesis of AD. However, its physiological functions have attracted less attention. Several studies have suggested that Aβ could play a neuroprotective or neurotrophic role at low concentrations (Giuffrida et al 2009, Whitson et al 1989). Low doses of Aβ could enhance learning and memory through facilitating induction and maintenance of LTP (Morley et al 2010). A small increase in Aβ levels by suppression of extracellular Aβ degradation

could lead to increased release of synaptic vesicles and enhanced neuronal activity in neuronal culture (Abramov et al 2009). Furthermore, increased Aβ levels may promote spontaneous excitatory postsynaptic currents without changing the inhibitory currents (Abramov et al 2009). Another study found that application of synthetic $A\beta_{42}$ at picomolar concentrations potentiated synaptic transmission, but at nanomolar concentrations $A\beta_{42}$ depressed synaptic transmission (Puzzo et al 2008). However, Aβ-induced synaptic effects are dependent on an optimal concentration of Aβ, and higher or lower concentrations lead to synaptic transmission dysfunction (Abramov et al 2009). This is also supported by other reports in which abnormal reduction of Aβ levels in mice deficient for APP (Seabrook et al 1999), PS1 (Saura et al 2004) or BACE1 (Laird et al 2005) were correlated with synaptic transmission dysfunction. Thus, Aβ may have an important physiological function in the brain. However, more work will be needed to determine whether effects that are seen are due to physiological or pathophysiological actions of Aβ.

1.3 Biology of amyloid precursor protein

1.3.1 Structure of the APP family

Amyloid precursor protein (APP) is a type I integral membrane protein and widely expressed in the mammalian brain (Kang et al 1987). The human APP gene, which is located on the long arm of chromosome 21, spans about 240 kb and consists of 18 exons (Lamb et al 1993, Yoshikai et al 1990). The Aβ encoding region is found in the 16^{th} and 17^{th} exons (Lemaire et al 1989, Suh & Checler 2002). APP undergoes alternative mRNA splicing to generate 8 different isoforms of APP comprising from 365 (APP365) to 770 (APP770) amino acid residues (Jacobsen et al 1991, Kitaguchi et al 1988). APP770, APP 751 and APP695 are the most common isoforms expressed

either in brain or peripheral tissues. APP770 and APP751, which contain a 56 amino-acid Kunitz protease inhibitor domain (KPI), are abundantly produced in glial cells (Donnelly et al 1988, Palmert et al 1988). APP770 differs from APP751 by the insertion of a 19 amino-acid residue OX-2 domain adjacent to the KPI domain of APP751. The OX-2 domain is only present in the APP770 isoform (Donnelly et al 1988). The shorter isoform, APP695, is normally produced in neurons, while less is produced in non-neuronal cells (Haass et al 1991). APP695 is the primary source of APP in the brain (Palmert et al 1988, Sisodia et al 1993). Interestingly, expression of APP751/770 isoforms is dramatically increased in astrocytes and microglia after injury (Siman et al 1989) and a substantial increase in APP695 expression can be detected during neuronal differentiation (Zheng & Koo 2011). However, the mechanism that regulates the expression of alternative APP isoforms is still poorly understood.

Two APP homologues have has been identified in mammals: the amyloid like protein-1 (APLP1) and the amyloid like protein-2 (APLP2) (Wasco et al 1992, Wasco et al 1993). Generally, APLP1 is restricted to neurons whilst APP and APLP2 are ubiquitously expressed in all tissues (Zheng & Koo 2011). The APP family members have a relatively conserved structure with a longer N-terminal extracellular region (ectodomain), a single membrane-spanning domain and a shorter C-terminal intracellular domain (cytoplasmic domain) (Dyrks et al 1988).

The extracellular region of APP contains several structural domains for which the crystal structure is available (Fig. 1.2). These are the E1 domain and the E2 domains (Dahms et al 2010, Rossjohn et al 1999). The E1 domain, which is close to the N-

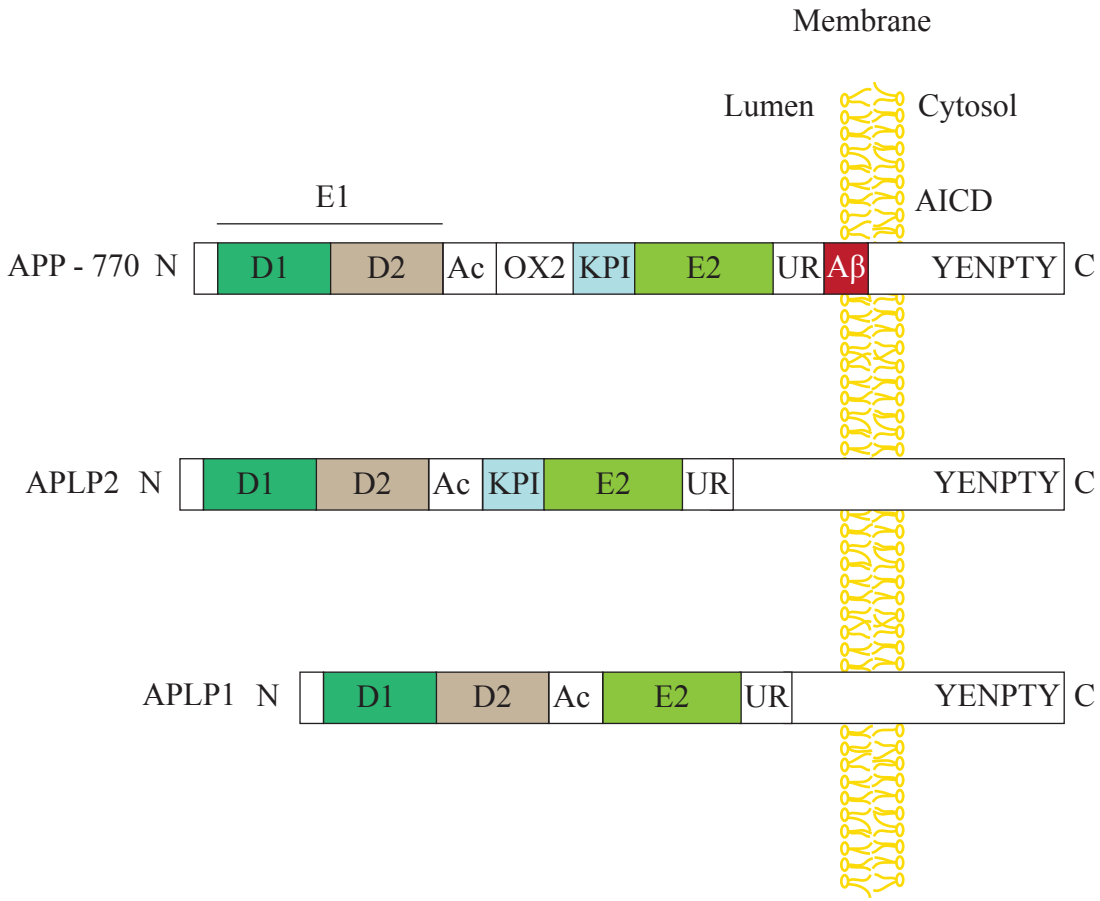

Figure 1.2 Domain structure of APP and its homologues. D1, heparin - binding domain; D2, metal binding domain including copper - binding domian and zinc - binding domian; Ac, acidic region; KPI: Kunitz-type protease inhibitor domain; E2: heparin binding domain; UR: unstructured region. YENPTY: adaptor protein binding region.

terminus of APP, contains a heparin binding domain (HBD) that forms a part of a growth factor like domain (GFLD) rich in cysteine (Small et al 1994), and a metal (copper and zinc) binding domain (Bush et al 1993, Hesse et al 1994, Reinhard et al 2005, Rossjohn et al 1999). Between the E1 domain and the E2 domain, there is an acidic region that is rich in aspartic and glutamic acid, and the KPI domain. The KPI domain is not found in APLP1. However, the OX-2 domain is located between the acidic region and KPI domain in APP770. The E2 domain is closer to the C-terminus and comprises another HBD/GFLD region and an unstructured region (UR) that contains α- and β- secretase cleavage sites (Mok et al 1997). The ectodomain of APP is followed by the transmembrane domain that has the γ-secretase cleavage site (Dyrks et al 1988, Weidemann et al 2002). The cytoplasmic domain of APP contains of a YENPTY motif that is proposed to be involved in transcriptional regulation and to interact with adaptor proteins (Cao & Sudhof 2001, Sabo et al 2003). Like the E1 and E2 domains of the extracellular region, the YENPTY motif of the intracellular region is highly conserved in APP family members (Jacobsen & Iverfeldt 2009). However, the Aβ sequence is not conserved. It is only present in APP, but not in APLP1 and APLP2 (Kang et al 1987).

1.3.2 APP trafficking and processing

After expression, APP is translocated into the endoplasmic reticulum (ER) and is post -translationally modified in the Golgi apparatus by glycosylation, sulfation, phosphorylation and palmitoylation (Bhattacharyya et al 2013, Selkoe 2001). Immature APP species undergo N- and O- glycosylation in the Golgi (Oltersdorf et al 1990, Weidemann et al 1989) where the N- and O- glycosylated mature APP is then trafficked to the cell surface (Koo et al 1996). At the cell surface, APP can be

internalized through clathrin-induced endocytosis via interactions with the YENPTY motif in the cytoplasmic domain of APP (Yamazaki et al 1996). After internalization, APP is delivered to the endosome, after which it can be recycled back to the cell surface, or it can be delivered to the lysosome where it can be further degraded (Perez et al 1999, Yamazaki et al 1996).

APP undergoes γ-secretase cleavage after the ectodomain shedding by α or β cleavage (Sisodia 1992). The α-secretase cleavage of APP to produce sAPPα may occur at the cell surface (Koo et al 1996), but some α-secretase cleavage may occur in the secretory and endocytic vesicles (Parvathy et al 1999, Tomita et al 1998). The β-site APP cleaving enzyme-1 (BACE1), which is the β-secretase (Cai et al 2001, Sisodia et al 1990), is mainly distributed in membranes of the Golgi and endosomes (Koo & Squazzo 1994). This suggests that APP can be cleaved in both secretory and endocytic steps. Therefore, β-secretase processing and Aβ production may commonly occur in acidic compartments in which β-secretase is most activated (Koo & Squazzo 1994, Vassar et al 1999). The site of intracellular γ-secretase cleavage of APP has not been well characterized, but several studies indicate that the γ-secretase complex is located, and has activity in, multiple cell compartments including the ER, Golgi, cell membrane and endosomal/lysosomal system (Buxbaum et al 1988, Kaether et al 2002, Pasternak et al 2004, Tarassishin et al 2004).

APLP1 and APLP2 are thought to undergo similar proteolytic processing to APP (see section 1.2.1) although they lack the Aß peptide region. Soluble forms of APLPs could be detected in human brain and cerebrospinal fluid (Webster et al 1995). In addition, Eggert (et al 2004) found that both homologues of APP were cleaved via α

and ß secretase like-pathways. Furthermore, p3 and Aß-like fragments of APLP2, and a p3-like fragments of APLP1, were detected in the conditioned medium of stably transfected SH-SY5Y cells (Eggert et al 2004, Minogue et al 2009). Moreover, increased levels of soluble APLP2 and its C-terminal fragments were found in ADAM10 (α-secretase) over-expressing transgenic mice (Endres et al 2005). APLP1 processing was reported to be independent of BACE 1 (β-secretase) reactivity because BACE1 inhibitors had no effect on APLP1 shedding (Eggert et al 2004, Minogue et al 2009). However, a recent study on BACE1- knockout and BACE1- overexpressing mice showed that BACE 1 tightly regulates the level of full length and soluble APLP1 and APLP2 (Sala Frigerio et al 2010). Deficiency in a functional part of γ-secretase (presenilin1 (PS1)), not only leads to failure of Aβ secretion in neurons, but also causes accumulation of C-terminal fragments of APP and APLP1 in neurons (Naruse et al 1998). These studies support the view that processing of APP homologues is similar to that of APP.

1.3.3 APP secretases

APP is cleaved by proteases, called secretases. Three APP secretases have been designated as the α-, β- and γ-secretases.

1.3.3.1 α - Secretase

The "non-amyloidogenic" pathway of APP processing involves α-secretase cleavage. This pathway is the primary pathway through which APP undergoes constitutive shedding. APP is cleaved within the Aβ sequence by α-secretase on the C- terminal side of amino acid residue 16 of the Aβ sequence (Sisodia 1992, Sisodia et al 1990). Thus, the primary route of APP processing precludes the generation of intact Aβ. The

enzymes that participate in α-secretase cleavage are members of the disintegrin and metalloproteinase (ADAM) family. Tumor necrosis factor-α converting enzyme (TACE) was the first identified ADAM, and is known as ADAM17 (Buxbaum et al 1998). One year after the identification of ADAM17 as an α-secretase, ADAM9 and ADAM10 were also reported to function as α-secretases (Koike et al 1999, Lammich et al 1999). ADAM10 overexpression leads to a major increase in α-secretase cleavage of APP (Lammich et al 1999, Postina et al 2004), a result that was later confirmed by Kuhn et al (2010). In addition, ADAM17-knockout mice show reduced production of sAPPα, although small inhibitory RNA (siRNA) mediated knockdown of ADAM17 did not show a reduction in α-secretase dependent APP processing (Buxbaum et al 1998, Kuhn et al 2010). However, mice with an ADAM9 deletion had neither major abnormalities nor a significant difference in α-secretase associated APP processing (Kuhn et al 2010, Weskamp et al 2002). Therefore, the major constitutive α-secretase activity may be contributed by ADAM10, with ADAM9 and 17 are more likely only to be involved only in regulation of α-secretase cleavage (Kuhn et al 2010, Moss et al 2011).

Besides APP processing, ADAMs play a physiological role in various types of cellular processing. ADAMs are suggested to act as sheddases that catalyze the release of soluble extracellular domains from proteins (Huovila et al 2005). This process is essential for the release of cytokines and growth factor ligands. For example, ectodomain shedding by ADAM10 can lead to release of epidermal growth factors (EGFs) and β cellulin (Sahin et al 2004). A recent paper indicates that senescence-associated release of transmembrane proteins requires proteolytic processing by ADAM17 (Effenberger et al 2014). ADAM17 can regulate the release

of tumour necrosis factor-α by acting as a sheddase (Black et al 1997). ADAMs-induced ectodomain shedding participates in cellular processing of transmembrane proteins, thus ADAMs may cleave APP to produce soluble APP fragments that play a role in downstream cellular signalling (Demars et al 2011, Hoey et al 2009, Huovila et al 2005).

1.3.3.2 β-Secretase

In the amyloidogenic pathway, APP is cleaved by β-secretase at the N-terminal end of the Aβ sequence to produce the soluble fragment sAPPβ, and a membrane bound fragment C99 which can be subsequently cleaved by γ-secretase to produce Aβ. The β-site APP cleaving enzyme 1 (BACE1) is a transmembrane aspartic protease that has all the characteristics of a β - secretase (Hussain et al 1999, Lin et al 2000, Vassar et al 1999, Yan et al 1999). BACE1 is primarily expressed in the brain and contributes the essential role of a β-secretase (Hussain et al 1999, Lin et al 2000). A homologue of BACE1, known as BACE2, can cleave APP in the middle of the Aβ domain and is expressed widely in peripheral tissues, but has a lower expression levels in brain than BACE1 (Fluhrer et al 2002, Lin et al 2000).

BACE1 has two aspartate domains that form the active site; mutation of either aspartate residue leads to inactivation of BACE1 (Hussain et al 1999, Knops et al 1995). In AD patients, both the expression and activity of BACE1 is elevated (Fukumoto et al 2002, Holsinger et al 2006, Yang et al 2003). BACE1 was reported to be the enzyme that contributes to the majority of Aβ production by neurons, and BACE1 knockout (BACE1 KO) mice show no significant neuronal Aβ and sAPPβ production (Dominguez et al 2005, Luo et al 2001). BACE2 KO mice also show a normal healthy phenotype, but mice with a double knockout of BACE1 and BACE2

display an increased incidence of lethality phenotype (Dominguez et al 2005). BACE1 deficiency did not affect Aβ production in glia, supporting the idea that BACE2 may be responsible for Aβ generation in glia in the brain of AD patients (Dominguez et al 2005). Thus, both BACE1 in combination with BACE2 may be needed for the maintenance of a healthy phenotype and normal APP processing.

BACE1 KO mice do not exhibit a major phenotypic difference to wild type mice (Roberds et al 2001). In addition, deletion of the *BACE1* gene rescues the memory deficiency in APP transgenic mice (Ohno et al 2006). This suggests that inhibition of BACE1 activity is a viable strategy for AD therapy. However, further studies have found that cognitive and emotional abnormalities, and synaptic dysfunction and spatial memory deficits appear in BACE1 KO mice (Laird et al 2005). Furthermore, studies on both BACE1 KO and over-expressing mice indicate that BACE1 contributes to myelination (Hu et al 2006, Willem et al 2006). Moreover, electroencephalographic recordings reveal that kainic acid-induced seizures occur more frequently in BACE1 KO mice than in wild type mice (Hu et al 2010). BACE1 can act as sheddase for a variety of substrates including neuregulin, the β-subunits of the voltage-gated sodium channels, interleukin-1 receptor 2, as well as the low-density lipoprotein (LDL) receptor-related protein (Cai et al 2012, Klaver et al 2010, Vassar et al 2009). Therefore, BACE1 plays a variety of roles in cellular processes, and it is thus essential to monitor the potential side effects of BACE1 inhibitor use as a therapeutic treatment for AD (Klaver et al 2010).

1.3.3.3 γ-Secretase

After ectodomain shedding by α- or β-secretases, the membrane bound fragments C99

and C83 are further cleaved by the γ-secretase to release AICD, Aβ or p3. γ - Secretase is a transmembrane protein complex that contains at least four protein subunits, including presenilins (PS1 or PS2), anterior pharynx-defective 1 (APH-1), presenilin enhancer 2 (PEN 2) and nicastrin (NCT) (Kimberly et al 2003b). The formation of the γ-secretase complex occurs in sequential steps, with APH-1 and NCT initially forming a stable complex with PS, followed by the addition of PEN 2 to form the active γ - secretase (Hu & Fortini 2003, LaVoie et al 2003).

All the components (PSs, APH-1, PEN 2 and NCT) are important for stability, maturation and activity of γ-secretase (Francis et al 2002, Goutte et al 2002, Kimberly et al 2003b). Deletion of a conserved hydrophilic domain of nicastrin inhibits γ-secretase and thereby inhibits Aβ production (Shirotani et al 2003, Yu et al 2000). Deletion of APH-1 causes a reduction in PS levels (Lee et al 2002, Shirotani et al 2004) whereas overexpression of APH-1 can cause accumulation of PS (Takasugi et al 2003). Therefore, APH-1 may act as a cofactor for PS that contributes to γ-secretase activity. PEN-2, a membrane-spanning component of the γ-secretase complex, coordinately regulates PS1 proteolytic processing (Luo et al 2003, Steiner et al 2002).

The PSs are suggested to be the major catalytically active components of γ-secretase (De Strooper et al 1998, Figueroa et al 2002, Wolfe et al 1999). Mammalian presenilins have two family members, presenilin 1 (PS1) and presenilin 2 (PS2). Cleavage of α- and β-secretase is not affected by *PS1* knockout, whereas cleavage by γ-secretase is completely blocked, causing accumulation of APP C-terminal fragments and a drop in Aβ levels (De Strooper et al 1998). PS mutations cause a

decrease in γ-secretase function. This leads to the incomplete digestion of the Aβ-peptide, which may contribute to increased vulnerability of the brain, and early onset of the inherited form of AD (De Strooper 2007).

Apart from APP, γ-secretase has several other substrates. One of the most notable substrates is the cell-surface receptor, Notch (De Strooper et al 1999). Cleavage of APP and Notch by γ-secretase appears to be similar (Kimberly et al 2003a). The Notch intracellular domain (NICD) is liberated by γ - secretase cleavage, resulting in its translocation to the nucleus where, it forms complexes with DNA binding proteins, and thereby acting as a regulator of downstream gene transcription (Jarriault et al 1995, Struhl & Adachi 1998). PS1 deficiency leads to a reduction in Notch proteolytic processing of Notch, and a decreased level of NICD. Thus the Notch signalling pathway is affected by PS1 (De Strooper et al 1999, Wong et al 1997). Notch signalling plays a variety of roles in CNS development including modulation of neural stem cell (NSC) proliferation, differentiation, maturation and survival (Lathia et al 2008). In addition, integrin regulates epithelial cell differentiation by modulating Notch activity (Gomez-Lamarca et al 2014). Therefore, inhibition of γ-secretase for the treatment of AD may lead to toxicity caused by reduced Notch signalling (De Strooper et al 1999).

1.3.4 Function of APP

The biological properties of APP have been studied intensively since its sequence was first reported in 1987 (Kang et al 1987). However, the physiological roles of APP still remain unclear (Zheng & Koo 2006). Studies on APP knockout (APP KO) mice suggest that APP may play a variety of interesting roles (Dinet et al 2011, Hung &

Selkoe 1994, Puig et al 2012). APP KO mice (Zheng et al 1996) display a number of physiological abnormalities (Magara et al 1999, Seabrook et al 1999, Steinbach et al 1998) compared to wild-type mice. However, it must be noted that the two homologous proteins, APLP1 and APLP2 may provide some functional compensation for APP deletion or deficiency (Heber et al 2000). Triple knockout of APP, APLP1 and APLP2 is lethal during embryonic development, suggesting a developmental function may be blocked when compensation by other family members is lost (Herms et al 2004). Taken together, various studies suggest that APP plays an essential role in the maintenance and normal physiology of the brain (Heber et al 2000, Zheng & Koo 2006). In this section, studies related to functions of APP in neurodevelopment are reviewed.

1.3.4.1 Role of APP in cell adhesion and neurite outgrowth

The E1 and E2 regions in the extracellular domain of APP have been shown to interact with extracellular matrix proteins: laminin (Kibbey et al 1993), collagen type 1 (Beher et al 1996) heparin sulfate proteoglycans (Mok et al 1997, Small et al 1994) and glypican-1 (Williamson et al 1996b), which suggests that APP plays a role in cell-substrate adhesion (Small et al 1999). APP and other family members can form homo- or hetero-dimers (Soba et al 2005), supporting the idea that APP is involved in cell- to cell contacts. Moreover, APP has been suggested to interact with proteins implicated in cell adhesion, such as integrins (Yamazaki et al 1997), neuron-glia cell adhesion molecule (Young-Pearse et al 2008), and transient axonal glycoprotein 1 (Ma et al 2008a). For example, APP and β1-integrin were colocalized on the cell surface at contact sites in neural cells (Yamazaki et al 1997). Soluble APP was shown to induce neurite outgrowth by interacting with β1-integrin (Young-Pearse et al 2008). siRNA mediated knockdown of APP expression causes defects in neuronal

migration that was associated with a cell adhesion deficiency (Young-Pearse et al 2007, Young-Pearse et al 2008).

The up-regulation of APP expression has been reported in growing neurites (Clarris et al 1995, Hung et al 1992), and down-regulation of APP seems to block neurite outgrowth (Allinquant et al 1995, Milward et al 1992). The effect of APP on neurite outgrowth may be driven by the physical cell adhesion characteristics of APP (Small et al 1994). APP was reported to bind to the neurite-promoting site of the transmemebrane glycoprotein, laminin to promote neurite outgrowth (Kibbey et al 1993). Furthermore, the APP heparin binding domain that is responsible for cell adhesion (Small et al 1999), is also suggested to be involved in regulation of neurite outgrowth (Small et al 1994). Indeed, APP-induced neurite outgrowth can be blocked by application of a peptide homologous to the heparin-binding region (Small et al 1994, Williamson et al 1996a). Moreover, hippocampal neurons grown on a substrate of APP transfected CHO cells, showed short-term neuronal adhesion and longer-term neurite outgrowth (Qiu et al 1995). Therefore, the APP-stimulated neurite outgrowth may be attributed to an increase in cell-cell or cell-extracellular matrix interaction.

Several other studies indicate that APP can directly or indirectly affect neurite outgrowth and axonal path finding (Gakhar-Koppole et al 2008, Hasebe et al 2013, Hoareau et al 2008, Young-Pearse et al 2008). For instance, the soluble forms of APP, sAPPα and sAPPβ, that are produced by α- and β-secretase cleavage respectively, can reportedly induce neurite outgrowth by binding to the p75 neurotrophin receptor (Hasebe et al 2013). The APP homologue, APLP2 was reported to have a role in the regulation of cell migration, neurite outgrowth and axonal path-finding (Thinakaran et al 1995). Thus, neurite outgrowth may be regulated by both APP and APLP2.

1.3.4.2 Role of APP in synaptogenesis and synaptic plasticity

APP is also suggested to participate in the regulation of synaptogenesis and synaptic plasticity. During development, the mitral cells of the olfactory bulb show dramatically increased in APP expression at a particular stage when the mitral cell dendrites are contacted by approaching neurites from olfactory receptor neurons (Clarris et al 1995). In neurons, APP is rapidly transported to the synaptic terminals after synthesis (Koo et al 1990, Moya et al 1994, Sisodia et al 1993) and its expression is predominantly up-regulated during the critical period of synaptogenesis (Clarris et al 1995, Wang et al 2009).

Synaptogenesis defects in APP KO mice may lead to a functional deficiency in neurotransmission and to long-lasting effects on synaptic plasticity and synaptogenesis (Priller et al 2006, Ring et al 2007, Zheng et al 1996). These defects may cause deficits in grip strength and locomotor activity in mice lacking APP (Ring et al 2007). For example, an in vitro study showed that deletion of APP can increase the number and cumulative strength of excitatory synapses during synaptogenesis (Priller et al 2006, Steinbach et al 1998), which may contribute to a hypersensitivity to kainate-induced seizure. APP-null mediated behavioural abnormalities are rescued by application of the soluble ectodomain form of APP, supporting the idea of a role for sAPP in synaptic function (Ring et al 2007). Endogenous Aβ is also proposed to be necessary for hippocampal synaptic transmission and plasticity at certain concentrations (Puzzo et al 2011, Puzzo et al 2008). Such as role may be one explanation for the cognitive impairment that occurs in APP-null mice (Dawson et al 1999, Seabrook et al 1999).

APP KO mice usually display mild phenotypes, as the functional deficiency may be masked by compensatory changes in APLP expression (Heber et al 2000, Korte et al 2012). The APP homologue, APLP2 is abundant in axon terminals in glomeruli and is present in pre- and postsynaptic compartments in the olfactory bulb (Thinakaran et al 1995). Studies from double knockout $APP^{-/-}$ /$APLP2^{-/-}$ (dKO) mice show that the APP family of proteins are essential for neuromuscular junction (NMJ) synapse patterning-APP/APLP2 dKO mice show impaired neuromuscular formation, with a reduced quantity of synaptic vesicles and impaired synaptic transmission (Ring et al 2007). APLP1/APLP2 dKO mice lack presynaptic nerve terminal sprouting, although they show normal endplate patterning, which means that APLP2 may play an essential role at NMJ synapses that cannot be compensated for by APP (Klevanski et al 2014). Therefore, APP and APLP2 may have distinct and divergent roles at NMJ synapses (Klevanski et al 2014).

APP has also been implicated in synaptic plasticity and neurotransmission (Hoe et al 2009a, Puzzo et al 2008, Yang et al 2009a). APP was reported to modulate levels and activities of Ca(v)1.2 L type calcium channels and to affect short-term plasticity through the regulation of calcium currents in GABAergic neurons (Yang et al 2009a). APP was also found to exert an effect on excitatory synaptic transmission via a change in two ionotropic glutamate receptors, AMPA receptor (AMPAR) and NMDA receptor (NMDAR) trafficking (Hoe et al 2009a, Hoe et al 2012, Lee et al 2010). APP plays a role in expression of the GluA2 subunit of the AMPAR- GluA2, which in turn affects synaptic transmission and plasticity (Isaac et al 2007). Thus APP may affect synaptic function by altering GluA2 (Lee et al 2010). Moreover, APP also mediates

synaptic function through an alteration of cell-surface NMDAR expression (Hoe et al 2009a). The NMDR is a calcium channel, and calcium permeability of the synapse membrane is crucial for synaptic plasticity (Cousins et al 2009). However, it has been suggested that APP's effects on synaptic transmission may be partially mediated by endogenous Aβ produced by APP processing because certain concentrations of Aβ can potentiate synaptic transmission (Puzzo et al 2008).

1.3.4.3 Non- neuronal functions of APP

Several non-neuronal functions of APP and APLPs have been proposed. APP has been reported to participate in blood coagulation (Bush et al 1990, Smith et al 1990). The isoform of APP containing the KPI domain is produced and released by platelets (Bush et al 1990, Gardella et al 1990, Van Nostrand et al 1991a). After stimulation of coagulation, APP, sAPP and Aβ, which accumulate in α- granules of platelets, is released along with the blood clotting factors that are also stored in the α-granule vesicles (Blair & Flaumenhaft 2009, Bush et al 1990, Gardella et al 1990, Smith et al 1990, Van Nostrand et al 1991b). Due to the presence of the KPI domain, APP may act as a serine protease inhibitor to inhibit the activity of coagulation factor Xia which plays an essential role in the blood coagulation cascade (Scandura et al 1997, Smith & Broze 1992, Smith et al 1990, Van Nostrand et al 1990). Studies on APP KO and APP/APLP2 dKO mice suggest the possibility that APP is involved in the regulation of glucose and insulin homeostasis. APP KO mice have hypoglycaemia until adulthood, whereas the APP/APLP2 double knockout mice display hyperinsulinaemia and die 24 hours after birth (Needham et al 2008). In addition, enhanced expression of adipocyte APP has also been found in individuals with obesity, and this supports a role for APP in the development of adipose tissue inflammation and insulin resistance

in obesity (Lee et al 2009, Lee et al 2008b). Moreover, up-regulated APP expression has been identified in several types of cancer including oral squamous cell carcinoma, human pancreatic adenocarcinoma and colon cancer (Ko et al 2004, Venkataramani et al 2010). Interestingly, siRNA-mediated APP knockdown significantly reduces tumour cell growth. Thus, APP expression may be related to tumour cell growth (Venkataramani et al 2010). The fact that APP has a trophic role and can promote cell proliferation (Ayuso-Sacido et al 2010, Saitoh et al 1989) is consistent with this idea. However, another study shows that the intracellular domain of APP may activate transcription of the tumour suppressor gene *P53* (Sumioka et al 2005).

1.3.4.4 APP signalling

Activation of specific cell signalling transduction pathways by APP is presumably important for APP to exert physiological effects, such as neurite outgrowth (Allinquant et al 1995), neural stem cell proliferation and differentiation (Clarris et al 1995, Hu et al 2013, Masliah et al 1992) and cell viability (Murayama et al 1996). However, the mechanisms underlying these effects have not been well clarified. In this section, the role of APP as a putative cell-surface receptor is discussed, as is role of the intracellular domain of APP (AICD).

1.3.4.4.1 APP may act as a cell -surface receptor

APP has been proposed to be a cell-surface receptor, because it shares in structural, post-translational modification and proteolytic processing similarities to Notch, a cell surface receptor involved in cell growth (De Strooper et al 1999, Selkoe & Kopan

2003). APP is also proposed to be a G-protein coupled receptor in a ligand-dependent and ligand-specific signalling (Okamoto et al 1995). Furthermore, APP has been reported to activate serine/threonine kinases and to stimulate the mitogen activated protein kinase (MAPK) pathway that transfers signals from the cell surface to the nucleus (Murayama et al 1996). In addition, the extracellular matrix protein, reelin binds the E1 domain of APP, causing a reduction in Aβ production and promoting neurite outgrowth (Hoe et al 2009b, Hoe et al 2006). Moreover, the diffusible molecule netrin-1 reportedly can interact with APP and participate in the regulation of Aβ production (Lourenco et al 2009). More recently, APP was shown to act as a co-receptor in netrin-1 mediated neural navigation and commissural axon outgrowth (Rama et al 2012). Although APP interacts with extracellular matrix proteins, the idea that APP may act as a cell-surface receptor is supported by that F-spondin's ability to bind to the E2 domain of APP, APLP1 and APLP2 (Ho & Sudhof 2004). As a signalling glycoprotein secreted by neurons, F-spondin may exert effects on neuronal development and repair (Peterziel et al 2011). However, binding of F-spondin to APP can lead to blockage of β-secretase cleavage. Therefore, F-spondin is proposed to regulate APP processing (Ho & Sudhof 2004). Although several physiological ligands can interact with APP, APP induced intracellular signalling transduction is the strongest evidence for the idea that APP is a cell-surface receptor (Dawkins & Small 2014).

1.3.4.4.2 Role of APP in intracellular signalling

APP is a substrate for regulated intramembrane proteolysis. This is a mechanism that regulates membrane protein activity, and has been implicated in a wide range of biological processes (Brown et al 2000, Lichtenthaler & Steiner 2007). The C-

terminal region of proteins produced by the γ-secretase translocate to the nucleus and activate gene transcription (Ebinu & Yankner 2002). The intracellular domain of APP (AICD) is suggested to translocate to the nucleus, where it regulates transcriptional activation (Cupers et al 2001, Hebert et al 2006, Lichtenthaler et al 2011), although AICD easily undergoes degradation under normal conditions (Kimberly et al 2001). However, AICD may be stabilized by interaction with adaptor proteins (Kimberly et al 2001, Small et al 2005). AICD can be produced in different forms because the ε -cleavage by γ -secretase generates AICD fragments that start further towards the C-terminus (Sastre et al 2001). Caspase-dependent AICD cleavage starts at a C-terminal position upstream of the ε- cleavage site (Lu et al 2000).

There are three sequence motifs in AICD that have been suggested to have functional significance. The first one is the ^{653}YTSI sequence, that is proposed to contribute to basolateral sorting of APP (Lai et al 1998), and participates in tyrosine-mediated and clathrin-based endocytic sorting (Bonifacino & Traub 2003). The second region, the ^{667}VTPEER sequence motif of AICD, has been implicated in certain pathophysiological processes. For example, Thr668 in the sequence was found to undergo increased phosphorylation in AD patients (Lee et al 2003). Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1), a prolyl isomerase, may affect the turnover of APP by halting GSK3β-induced phosphorylation at Thr668, as Pin1 overexpression reduces Aβ whereas a knockout of Pin1 results in an enhanced Aβ yield (Ma et al 2012a, Pastorino et al 2006). The third functional sequence motif, YENPTY, has drawn the most attention and been studied intensively. The YENPTY region has been shown to interact with many adaptor proteins that have phosphotyrosine binding (PTB) or phosphototyrosine interacting domains (PID)

(Borg et al 1996). APP homologues all contain the YENPTY motif, which means that all APP family members may interact with similar adaptor proteins and may function similarly as transcriptional regulators (Bressler et al 1996, Zheng & Koo 2011). This is consistent with the evidence for functional redundancy among APP homologoues from APP knockout mice studies.

The YENPTY motif has been shown to be bind to a variety of adaptor proteins: X11 family proteins (Borg et al 1996), Fe65 (Fiore et al 1995), Disable-1 protein (Homayouni et al 1999), JNK interacting protein 1 (Scheinfeld et al 2002b), ShcA/C (Tarr et al 2002) and growth factor receptor-bound protein 2 (Zhou et al 2004). Fe65 was the first identified APP binding partner (Fiore et al 1995) and has been investigated intensively. Fe65 binds to the YENPTY motif in a manner that is independent of tyrosine phosphorylation (Scheinfeld et al 2002a). Fe65 overexpression results in enhanced plasma membrane translocation of APP, and increased production of sAPPα and Aβ (Sabo et al 1999). This demonstrates that the interaction of APP with FE65 probably affects APP processing and trafficking. However, FE65 expression may lead to inhibition of APP maturation as well as a reduction in Aβ production when thr668 has been phosphorylated (Ando et al 2001). Therefore, FE65 may bind to both ^{667}VTPEER and YENPTY to mediate FE65-dependent gene transactivation (Cao & Sudhof 2001, Sumioka et al 2005).

The interaction of FE65 with AICD can recruit another protein, TIP60, and this AICD/FE65/TIP60 complex is suggested to be involved in transcriptional activation of several target genes (Kim et al 2003, Pardossi-Piquard et al 2005, Zhang et al 2007b). Studies of AICD's role in cell proliferation indicate that the

AICD/FE65/TIP60 complex may work as a negative regulator of neural stem/progenitor cell (NSPC) proliferation via down regulation of the epidermal growth factor receptor (EGFR) mediated gene transcription (Ayuso-Sacido et al 2010, Zhang et al 2007b). In addition, genes that participate in cell death and apoptosis, such as P53, are suggested to be a target of AICD-dependent activation (Alves da Costa et al 2006, Checler et al 2007, Ozaki et al 2006). Cell death and apoptosis occur in transfected cells expressing AICD (Konietzko 2012, Lu et al 2000), while those expressing mutant versions that contain no FE65 binding domain show little effect on cell survival. These findings demonstrate that the interaction of AICD with FE65 may be essential for mediating P53 - induced cell death (Konietzko 2012).

Apart from targets on EGFR or P53, AICD has also been reported to form multiple protein complexes affecting *APP* gene transcription (von Rotz et al 2004). In addition, the intracellular domains of APP and APLP are implicated in the regulation of neprilysin, which is an Aβ-degrading enzyme (Pardossi-Piquard et al 2005). Furthermore, APP and APLP2 have been shown to regulate cholesterol metabolism via AICD nuclear signaling that may involve modulation of the lipoprotein protein receptor 1 (LRP1) promoter (Liu et al 2007).

1.4 Risk factors for AD

AD can be separated into forms that can be distinguished according to the age of onset or their heritability. Early-onset AD (EOAD) is generally a familial AD (FAD), and presents before 65 years of age. Late-onset AD (LOAD) is generally not inherited, and is sometimes called sporadic AD (sAD), although this term may not be entirely appropriate. It usually occurs over the age of 65. FAD accounts for up to 5%

of AD cases, while LOAD contributes to the majority (~ 95%) of AD cases (Reitz et al 2011). Genetic studies suggest that an increase in Aβ production may be the cause of all forms of EOAD (Citron et al 1992, Duff et al 1996), whereas LOAD may be associated with a defect in removal of Aβ from the brain (Miners et al 2008), although this is not yet clearly demonstrated. Apart from Aβ production, metabolism as well as clearance, education, diet, physical activity, smoking, alcohol, and drugs are also suggested to correlate with the development of AD (Cummings et al 1998, Lannfelt 1996).

1.4.1 Familial AD (FAD)

FAD is an autosomal dominant disorder. Three genes have been firmly identified as responsible for the pathophysiology of FAD, with APP and presenilin genes (PS1and PS2) all causing changes in APP processing and Aβ production. These three genes have been found to bear AD linked mutations that are diagnostic disease biomarkers given the very high penetrance (>85%) and mostly autosomal dominant inheritance (Reitz et al 2011).

1.4.1.1 APP mutations

FAD mutations in APP occur within or near the Aβ encoding region, or close to the cleavage sites of the three secretases. All known mutations account for <0.1% of AD cases (Ancolio et al 1999, Eckman et al 1997, Goate et al 1991, Reitz et al 2011). Most dominantly inherited APP missense mutations may lead either to an increase in total Aβ production or to an increased ratio of $A\beta_{42}/A\beta_{40}$, which accelerates progression of AD (Lichtenthaler et al 2011, Reitz et al 2011, Selkoe 2001).

Mutations in APP co-segregate with FAD. These mutations include those known as Dutch (E693Q) (Levy et al 1990), London (V717I) (Goate et al 1991), Indiana (V717F) (Murrell et al 1991), Swedish (K670N/M671L) (Mullan et al 1992), and Arctic (E693G) (Nilsberth et al 2001). The Swedish double mutation occurs close to the β-secretase cleavage position and results in approximately 6-fold more Aβ generation compared to cells carrying the normal APP sequence (Citron et al 1992, Reaume et al 1996). APP that harbours mutations around the α-secretase cleavage site lowers α-secretase cleavage (Sahlin et al 2007) and results in more APP available to undergo β-secretase cleavage processing. Thus, Aβ production is elevated. γ-secretase cleavage site-related mutations are proposed to increase production of longer and more neurotoxic forms, such as $A\beta_{42}$ (Suzuki et al 1994). In addition, a mutation at codon 715 of the APP770 gene that has been identified in an Indian family with FAD, was reported to decrease $A\beta_{40}$ production without altering $A\beta_{42}$ yield. Hence, the ratio of $A\beta_{42}$ to the total amount of Aβ seems essential for AD development (Ancolio et al 1999).

1.4.1.2 Presenilin mutations

The presenilins form the main catalytic centre of γ-secretase and functionally participate in the γ-secretase-induced proteolytic cleavage of APP (Ahn et al 2010, De Strooper et al 1998, Figueroa et al 2002, Octave et al 2000). Mutations leading to FAD are found in the *PS1* and *PS2* genes, located on chromosome 14 and 1, respectively (Levy-Lahad et al 1995a, Levy-Lahad et al 1995b, Sherrington et al 1995). To date, approximately 180 different AD-linked polymorphisms in the *PS1* gene from 401 families have been characterized, but only 34 AD-associated mutations from 23 families have been found in the *PS2* gene

(http://www.alzforum.org/mutations/search/genes). Some *PS* gene mutations are proposed to be associated with the more rapidly progressing and aggressive forms of AD (Selkoe 2001).

Single-nucleotide substitutions have been suggested to account for the majority of AD-associated PS1 and PS2 mutations (Reitz et al 2011). Several reports indicate that point mutations in the *PS* gene lead to elevated production of $A\beta_{42}$, which contributes to the symptoms of FAD (Bentahir et al 2006, Duff et al 1996, Scheuner et al 1996). While PS mutations exert an apparent gain-of-function effect on γ -secretase activity, De Strooper (2007) suggests that the role of presenilin mutations involve a loss or decrease of γ-secretase function. A decrease in γ-secretase leads to the incomplete digestion of Aβ-peptide, which may contribute to an increased vulnerability in the brain, and thus explain the early onset of the inherited form of AD (De Strooper 2007).

1.4.2 Late onset AD (sporadic AD)

Genetic risk factors for FAD exhibit clear influences on AD symptom development, however known genetic risk factors for sporadic AD do not display clear effects on AD progression. The precise mechanisms by which genetic risk factors lead to AD remain poorly understood (Finelli et al 2014). Several gene variations have been identified as contributing to LOAD risk. Over 20 common genetic variants linked to sporadic AD have been identified through genome-wide association studies (GWAS) (Chouraki & Seshadri 2014). The top ten AD risk genes are ranked and shown in Table (1.1). Several genes are implicated in lipid transportation and metabolism, neuroinflammation, cytoskeleton dynamics as well as endocytosis (Olgiati et al 2011).

Interestingly, down-regulation of any of the proteins expressed by these genes does not affect Aβ production, implying that the expression of these proteins may contribute to AD via a pathway unrelated to enhancement of Aβ production (Bali et al 2012).

1.4.2.1 Apolipoprotein E (apoE)- the major risk factor

The most prevalent genetic risk factor for LOAD is the **ε-**4 allele of the *apolipoprotein E* (*APOE ε4*), which is found in more than 50% of AD patients (Michaelson 2014). ApoE consists of three allelic variants that are determined by cysteine-to-arginine substitutions at positions 112 and 158 of the amino-acid sequence (Weisgraber et al 1981). The variants are named as apoE 2 (cys112, cys158), apoE 3 (cys112, arg158), and apoE 4 (arg112, arg158) which correspond to the alleles ε2, ε3 and ε4 respectively (Zannis et al 1982). The small difference in amino - acid sequence gives rise to great differences in the structure and function of the isoforms (Mahley et al 2006). The ε - 4 allele is more frequently found in AD cases. People carrying two copies of the alleles have up to 12–15 fold increased risk of developing sporadic AD compared to non-carrier subjects. Even one copy of the ε4 allele increases the risk of AD threefold (Bertram et al 2007).

ApoE is the major apolipoprotein expressed in brain. ApoE is also the constituent of several lipoproteins, such as the high and very low density lipoproteins and chylomicrons (Chouraki & Seshadri 2014). In the CNS, apoE is produced mainly in astrocytes and to a lesser extent in microglia (Grehan et al 2001, Pitas et al 1987). Production of apoE in neurons is normally is low, but can occur under certain

physiological and pathological conditions (Xu et al 1999). ApoE-containing lipoproteins are responsible for transport and delivery of CNS lipids and cholesterol to cells (Holtzman et al 2012), and for the activation of neuroinflammation (Lynch et al 2001). They also act as a ligand in receptor-mediated endocytosis of lipoprotein particles, such as in the uptake of cholesterol needed to support synaptogenesis and the maintenance of synaptic connections (Pfrieger 2003a, Pfrieger 2003b).

ApoE has been shown to colocalize with amyloid deposits in both AD and CAA (Namba et al 1991, Wisniewski & Frangione 1992). A positive relationship between plaque density and apoE 4 dose in AD has been shown in post-mortem brain studies (Rebeck et al 1993, Schmechel et al 1993). ApoE may affect the quantity of Aβ accumulation and deposition as well as the Aβ42/Aβ40 ratio in an isoform-specific manner. ApoE4 is the most potent in this regard, and apoE2 the least potent (apoE4 > apoE3 >> apoE2) (Fagan et al 2002, Fryer et al 2005, Holtzman et al 2000). Indeed, individuals with apoE2 have a lower risk for AD (Corder et al 1994). In contrast, several papers suggest that apoE is able to inhibit Aβ aggregation based on in vitro studies (Evans et al 1995, Naiki et al 1997, Wood et al 1996a, Yang et al 1999). These opposite results may be due to differences in Aβ or the apoE preparation (Holtzman et al 2012).

ApoE isoforms have been implicated in Aβ clearance. Binding of apoE to soluble Aβ forms an apoE-Aβ complex that may be taken up via receptor-mediated endocytosis into neurons, astrocytes and microglia, after which it may be degraded in lysosomes (He et al 2007, Jiang et al 2008, Li et al 2012a, Yamauchi et al 2002, Yang et al 1999, Zhao et al 2014). Receptor-mediated cellular uptake of Aβ bound to apoE has been

Table 1.1 Genetic risk factors for LOAD

Gene	Protein	Potential Functions
ApoE 2/3/4	Apolipoprotein E	Lipid transport
BIN1	Bridging-integrator 1	Tumour suppressor and cell endocytosis
CLU	Clusterin	Lipid transport
ABCA7	ATP-binding cassette, sub-family A, Member 7	Lipid homeostasis
CR1	Complement receptor 1	Regulation of immune response
PICALM	Phosphatidylinositol-binding clathrin assembly protein	Clathrin-mediated endocytosis
MS4A6A	Membrane-spanning 4-domians, subfamily A, member 6A	Unknown, expected to have receptor activity
CD33	Myeloid cell surface antigen CD33	Myeloid lineage development. Immune response
MS4A4E	Membrane-spanning 4-domians, subfamily A, member 4E	Unknown, cell component
CD2AP	CD2-associated protein	Regulation of actin cytoskeleton

This table shows 10 top risk genes for LOAD, published on http://www.alzgene.org; ranked by the AlzGene database meta – analysis.

reported to occur through a low density lipoprotein receptor (LDLR) - LRP1 dependent pathway (Kang et al 2000, Yang et al 1999), and has been considered to be a possible mechanism of Aβ clearance. Alternatively, Aβ removal from the brain into the circulation may be modulated by apoE via trafficking across the blood brain barrier (BBB) (Bell et al 2007, Deane et al 2008). A weaker binding affinity of apoE4 for Aβ may lower Aβ clearance from the brain (Deane et al 2008). More abundant Aβ aggregation may therefore be caused by a decrease in Aβ clearance. Notably, a recent study suggests that apoE4 may negatively affect Aβ removal by competing with Aβ for clearance receptors rather than by specific binding to Aβ (Verghese et al 2013). In vitro studies show that Aβ can be directly internalized by cells through the LDLR, which is the major receptor for apoE (Basak et al 2012). ApoE has been shown to enhance protease-mediated Aβ degradation by neprilysin, but apoE 4 shows less efficiency in facilitating proteolysis of Aβ (Jiang et al 2008). Finally, a more recent study shows that apoE may be involved in the modulation of microglia activation, with apoE enabling the conversion of macrophages to the anti-inflammatory M2 type (Aguzzi et al 2013, Baitsch et al 2011). In this case, apoE4 may possibly be less effective in its anti-inflammatory action (Zhu et al 2012). Studies on mice argue a role of apoE in Aβ in clearance, as APP cross apoE knockout mice exhibits decreased amyloid deposition compared to APP mice with normal apoE (Bales et al 1997). Recently, another study also argues in favour of apoE having a function in Aβ removal-normal cognitive and neurological function, as well as normal CSF Aβ and tau proteins levels were described in a patient with a complete absence of apoE due to an APOE frame shift mutation (Mak et al 2014).

Although apoE is the main identified genetic risk factor for LOAD, neuropathological

alterations other than Aβ deposition, such as neuro-inflammation and synaptic plasticity, have also been found to associate with apoE, (Kuszczyk et al 2013, Zhu et al 2012). However, the precise mechanism by which apoE contributes to AD progression remains to be elucidated.

1.4.2.2 TREM2

Recent studies have identified a rare variant of the triggering receptor expressed on myeloid cells 2 (TREM2) as a significant risk factor for LOAD (Guerreiro et al 2013, Jonsson et al 2013). This unusual variant in the TREM2 gene has also been linked to EOAD in another study (Pottier et al 2013). A missense mutation (rs75932628) in the *TREM2* gene leads to a C to T base pair change that results in a histidine to arginine amino - acid substitution at position 47 (R47H) (Finelli et al 2014). This mutation and other mutations in TREM2 have been implicated not only in AD, but also in Parkinson's disease, frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) by other independent studies (Cady et al 2014, Cuyvers et al 2014, Feng et al 2014, Rayaprolu et al 2013).

The TREM2 gene located on chromosome 6 encodes a protein comprising 230 amino - acid residues (Paloneva et al 2002). TREM2, is a transmembrane glycoprotein that belongs to the immunuoglobulin (Ig) superfamily. The expressed protein has an extracellular domain that is followed by a membrane spanning domain that is joined to a cytosolic domain (Lue et al 2014a). TREM2 serves as an innate immune receptor that is expressed on the cell membrane of monocyte-derived dendritic cells like macrophages, osteoclasts as well as microglia in the CNS (Colonna 2003). Although generally thought of as a myeloid cell specific protein, TREM2 may be found in

neurons and possibly in oligodendrocytes (Guerreiro et al 2013, Kiialainen et al 2005, Sessa et al 2004). However, some studies have failed to detect TREM2 expression in oligodendrocytes (Chertoff et al 2013, Sessa et al 2004), thus warranting further investigation of its expression in this cell type.

Microglia significantly contribute to phagocytosis of apoptotic neurons and clearance of synapses during development and in adulthood (Miyamoto et al 2013, Neumann et al 2009, Wake et al 2011). In the CNS, TREM2 is expressed predominantly in microglia, and microglia have been found to scavenge apoptotic neurons via TREM2 without triggering inflammation (Takahashi et al 2005). Furthermore, microglial TREM2 is suggested to play an essential role in maintaining immune homeostasis of the CNS tissues (Neumann & Takahashi 2007). Therefore, gene variants of *TREM2* may be correlated with dysfunction of microglia.

AD pathological features such as mature and diffuse plaques have been reported in the patients carrying the R47H TREM2 mutation (Guerreiro et al 2013). Furthermore, post - mortem studies of AD brains and non-demented case controls reveal that elevated levels of TREM2 protein are associated with an increase in phosphorylated tau and the marker of apoptosis, activated caspase 3, as well as with the loss of the presynaptic protein SNAP25 (Lue et al 2014b). This result is seen in a transgenic mice model as well, with enhanced TREM2 mRNA and protein levels in microglia around plaques and neurons in APP transgenic mice brains compared to corresponding wild - type controls (Frank et al 2008, Guerreiro et al 2013). Microglia activation marker complement mRNA was detected in plaque-associated microglia as well (Melchior et al 2010). In addition, AD transgenic mice missing one *TREM2*

allele display no significant alteration in their amyloid plaque load, although they do exhibit a 40% reduction in microglia around amyloid plaques (Ulrich et al 2014). However, up-regulation of TREM2 in AD mice reduces amyloid plaque accumulation, attenuates neuroinflammation and decreases neuronal and synaptic loss (Jiang et al 2014). Enhanced Aβ phagocytosis has been observed in TREM2-overexpressing microglia (Lue et al 2014a). These results suggest that mutations in TREM2 may result in a loss of function leading to a deficiency in microglial-induced amyloid phagocytosis. However, further investigations are required to confirm this hypothesis.

1.4.2.3 CST3

Cystatin C (CysC), is a cysteine protease inhibitor that is found widely in CSF and other tissues (Turk et al 2000, Turk et al 2008). CysC is encoded by the *CST3* gene. A polymorphism in the CST3 gene resulting in an Ala/Thr transition (G73A) has been identified, which is reported to increase the risk of AD (Bertram et al 2007, Crawford et al 2000), although this idea has been controversial (Dodel et al 2002, Monastero et al 2005). Different findings may be due to the use of different ethnic groups. In addition, allelic variation at the same position may be another reason for opposing results, with either Ala (allele A) or Thr (allele B) of *CST3* being reported as responsible for AD development (Crawford et al 2000, Finckh et al 2000, Kaur & Levy 2012).

CysC has been found to deposit with Aβ in amyloid-loaded vascular walls (Maruyama et al 1990), and in the core of amyloid plaques in the AD brain (Levy et al 2001). Compared to non-demented cases, much lower CysC levels can be detected

in the CSF of AD patients (Hansson et al 2009, Simonsen et al 2007), although specific neuronal cells in AD patients display higher CysC expression (Deng et al 2001, Levy et al 2001). Apart from AD, alterations in CSF CysC concentration in CSF has been implicated in other neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS) (Pasinetti et al 2006, Tsuji-Akimoto et al 2009, Urbizu et al 2014, Yang et al 2009b).

There are different views on the role of CysC in AD. CysC may be involved in exacerbation of neurodegeneration, or it may served a neuroprotective role in response to pathological stimulation (Gauthier et al 2011, Kaur & Levy 2012, Maruyama et al 1990, Suzuki et al 2014b, Urbizu et al 2014). In vitro studies show that CysC has a protective function in neuronal cells, as cells from CysC knockout mice (Huh et al 1999) are more vulnerable to in vitro-toxicity compared to cells isolated from wild-type mice (Tizon et al 2010). A role of CysC in CAA-induced haemorrhage has been proposed because CysC co-deposits with Aβ, and may increase sensitivity to cerebral haemorrhage (Maruyama et al 1990). APP overexpressing mice that express human CysC show a diminished Aβ level compared to APP mice with normal CysC (Kaeser et al 2007, Mi et al 2007). However, CysC knockout mice crossed with APP overexpressing mice also show enhanced Aβ degradation (Sun et al 2008).

1.5 APP transgenic and knockout mice

Transgenic mouse models have been widely used in studies of AD pathogenesis, although there are still no mice carrying a single AD associated gene allele that develop all of the features of human AD (Ashe & Zahs 2010). However, APP

overexpressing mouse models have exhibited some of the lesions and symptoms of AD. APP knockout mice probably exhibit biological deficits caused by APP deficiency, and may provide valuable insights into the function of APP (Ashe & Zahs 2010, Duyckaerts et al 2008).

1.5.1 Transgenic mouse model of AD

Transgenic animal models of AD provide strong evidence that Aβ is the major cause of AD (http://www.alzforum.org/res/com/tra/default.asp). The PDAPP mouse bearing a single valine to phenylalanine substitution at residue 717 (Indiana mutation) (Murrell et al 1991) was the first APP mouse model that developed and exhibited similar neuropathology to AD, including Aβ deposition, neuritic plaques, synaptic loss, astrocytosis as well as microgliosis (Games et al 1995). The Tg2576 mouse has been used widely for studying AD pathogenesis (Frautschy et al 1998, Hsiao et al 1996, Seo et al 2010). This mouse expresses high levels of human APP with the double Swedish mutation ($APP_{K670N,/M671L}$) under the control of the hamster prion protein (PrP) promoter (Hsiao et al 1996). Apart from age-dependent changes in Aβ levels in the brain CSF, and plasma (Kawarabayashi et al 2001), Tg2576 mice have a 14-fold increase in Aβ42 levels and develop Aβ plaques, and display behavioural deficits and cognitive impairment after 9 months (Hsiao et al 1996). Moreover, Tg2576 mice also show Aβ-mediated microglial activation (Frautschy et al 1998), and aged Tg2576 mice exhibit severe motor neuron degeneration in the spinal cord (Seo et al 2010). Mice harbouring the FAD mutations in the *PS1* gene produce an elevated level of Aβ42, but do not make amyloid plaques (Duff et al 1996). However, mice with both *APP* and *PS* mutations develop aggressive amyloid pathology (Gordon et al 2001). The amyloid plaques that accumulate in APP/PS1 mice develop at a very early

stage - around 2 to 3 months (Gordon et al 2001). In addition, amyloid-associated pathology, including dystrophic synapses, hyperphosphorylated tau, gliosis, and increased microglia activity starts to appear from 1 to 8 months of age in APP/PS1 mice. Memory and cognitive deficits present at approximately 8 months of age (Arendash et al 2001, Radde et al 2006).

While APP transgenic mice exhibit Aβ plaques, it still remains unclear why they do not form neurofibrillary tangles (Radde et al 2008). However, transgenic mice bearing three mutations in APP, PS and tau respectively, progressively develop amyloid plaques and tangles and replicate certain aspects of AD neuropathology (Billings et al 2005, Oddo et al 2003). Nicotinic α7 receptors are lost selectively in the hippocampus and cortex and synaptic dysfunction is present prior to plaque and tangle pathology (Oddo et al 2005, Oddo et al 2003). Thus transgenic mice provide an opportunity to investigate some aspects of AD pathophysiology and to test certain therapies for AD, even though they do not recapitulate every pathological feature of the disease.

1.5.2 APP knockout mice

APP knockout (APP KO) mice were made by inactivation of the APP gene (Zheng et al 1996). APP KO mice are viable and fertile, and are relatively normal and healthy until 12 weeks of age (Zheng et al 1996). They exhibit decreased body weight (approximately 15%–20% smaller) and have a reduced brain weight (about 10% less) that is correlated with a reduced size of forebrain commissures. This supports the idea that APP may play a role in neurite outgrowth (Magara et al 1999, Zheng et al 1996). A deficiency in grip strength and a reduction in locomotor activity in APP KO mice (Zheng et al 1995) indicates a possible contribution of APP to neuronal or muscular

function. In addition, a profound reduction of proteins in the presynaptic terminal vesicle, and of dendritic proteins in the cortex and hippocampus, are observed in APP KO mice (Dawson et al 1999). This suggests that APP may contribute to maintaining synaptic function during ageing. In addition, an in vivo study describes a significant reduction in spine density, a decrease in the length of apical dendrites, and a decline in dendrite arborization in APP KO mice aged 12 to 15 months, but not at younger ages. This supports the suggestion that APP may be important for dendritic integrity and maintenance in the hippocampus (Tyan et al 2012).

Interestingly, to date, a mild somatic growth deficit is the only abnormality observed in APLP1-KO mice (Heber et al 2000), and no serious abnormalities have been described in APLP2-KO mice (von Koch et al 1997). The lack of phenotype may possibly be explained by functional redundancies of APP family proteins in transgenic mice (Muller & Zheng 2012). However, APLP2/APLP1 and APLP2/APP double knockout mice usually die shortly after birth (Heber et al 2000, von Koch et al 1997). In contrast, APLP1/APP-mice mice are viable and fertile, and have not been reported to have any additional abnormalities (Heber et al 2000). This suggests that there may not be compensation between APLP2 and other family members and it suggests a critical physiological role for APLP2 (Muller & Zheng 2012).

1.6 Current and prospective therapeutic approach for AD

At present, there are no clinically proven therapies that can block or even to delay progression of AD. Current therapeutic approaches only ameliorate the symptoms and arrest the rate of development of the disease's pathological symptoms.

1.6.1 Current therapeutic treatment for AD

1.6.1.1 Acetylcholinesterase inhibitors

Loss of cholinergic synapses has been considered for many years to be closely associated with AD because cholinergic neurons are profoundly lost in the basal forebrain of AD patients (Bartus et al 1982, Coyle et al 1983). Cholinergic neurons are essential for memory and concentration, as well as for other cognitive processes. Hence, improvement of AD symptoms can be achieved by prolonged cholinergic stimulation (Sarter & Bruno 2004). Acetylcholinesterase (AChE) is the enzyme that degrades acetylcholine in the synaptic cleft to halt cholinergic neurotransmission. Inhibition of AChE could extend the duration of cholinergic neuron transmission in the brain, leading to mild relief of AD symptoms (Giacobini 1998). Inhibition of AChE by three approved AChE inhibitors: donepezil, galantamine and rivastigmine has been found to decrease AD symptoms to a small extent in the short term (Prvulovic et al 2010, Wilkinson et al 2009). Unfortunately, drug resistance has been reported with AD progression and several side effects have been also reported (Takeda et al 2006). When moderate-to-severe AD patients were administrated a combination of donepezil and a N-methyl-Daspartate (NMDA) receptors antagonist, memantine, there was a lessening in several AD-associated symptoms, although the clinical relevance of this benefit is unclear (Riordan et al 2011).

1.6.1.2 Memantine

Chronic long-term activation of NMDA receptors causes pathological influx of Ca^{2+}, and may ultimately lead to excitotoxicity and neuronal cell death (Butterfield & Pocernich 2003, Dodd 2002, Holscher 1998). Aβ was reported to disrupt the glutamatergic system and Ca^{2+} homeostasis (Mattson et al 1992, Mattson et al 1999,

Parameshwaran et al 2008). Thus, NMDA receptor induced excitotoxicity is considered to be one of the possible causes of neurodegeneration in AD (Danysz & Parsons 2012, Mattson et al 1999, Parameshwaran et al 2008, Parsons et al 1998).

Memantine is the only NMDA receptor antagonist clinically used for AD treatment, and acts via effects on the glutamatergic system (Danysz & Parsons 2012, Reisberg et al 2003, Riordan et al 2011). Memantine has a modest effect in moderate to severe AD at therapeutic concentrations (Kornhuber & Quack 1995). However, it showed little effects in mild AD (Schneider et al 2011). Nevertheless, administration of memantine has been reported to reduce Aβ generation in neuroblastoma cells, primary cortical cells and in APP and mutant PS transgenic animals (Alley et al 2010). AD like neuropathology was reduced in transgenic mice treated with memantine (Martinez-Coria et al 2010). Thus, pharmacologic manipulation of the NMDA receptors alone, or alternatively in combination with cholinergic pathway modification, may be a supplemental treatment to relieve AD symptoms to a small extent (Doraiswamy 2003, Prvulovic et al 2010).

1.6.2 Prospective therapeutic strategies to delay disease progression

In recent years, several other therapeutic strategies have been proposed for the treatment of AD.

1.6.2.1 Aβ - based approaches

Aβ is thought to play a central role in AD pathogenesis. Hence targeting Aβ production, aggregation and removal may be of therapeutic benefit. To inhibit Aβ production, drugs that target APP processing by inhibition of secretase activities are

being developed. Studies have also reported that up-regulation of α-secretase in APP processing decreases Aβ yield and improves cognitive behaviour in transgenic mice, implying that this may be a viable treatment for AD (Caccamo et al 2006, Lin et al 1999). In addition, APP undergoes γ-secretase cleavage to release Aβ, thus selectively inhibiting γ-secretase activity is also considered to be a viable therapeutic strategy. The applied dose of γ-secretase inhibitor would have to be carefully controlled to avoid blocking important signaling pathways, such as those downstream of Notch (Bergmans & De Strooper 2010). BACE1 (β-secretase), the other enzyme that is involved in Aβ generation has multiple substrates in vivo (Hu et al 2006, Kim et al 2007a) but fewer than γ-secretase. Thus, modification of BACE1 activities without interfering with important physiological signaling may be a more promising potential therapeutic strategy for AD.

Inhibition of Aβ aggregation has been proposed to be a prospective viable AD treatment, and indeed cognitive benefits have been reported both in animals and in humans who were given inhibitors of Aβ aggregation (Adlard et al 2008, Lannfelt et al 2008, Olcese et al 2009)**.** However, one must be careful when considering AD by inhibition of Aβ aggregation because the exact Aβ aggregation species causing Aβ mediated neurotoxicity remains unknown. Blocking aggregation of Aβ could end up being more detrimental if small aggregates are more neurotoxic, or if aggregation inhibiting aggregation increases the formation of longer aggregates.

Inadequate removal of Aβ from the brain may contribute to amyloid accumulation in the CNS, thus therapeutic strategies aimed at increasing Aβ removal and clearance are being in investigated. They may be a more promising approach given that both β- and

γ-secretase inhibition may have off-target effects. For example, APP transgenic mouse studies indicate that increased astrocytic lysosome biogenesis may promote uptake, trafficking and degradation of Aβ, which suggests that activation of astrocytic lysosomes may facilitate Aβ removal and attenuate amyloid induced AD pathogenesis (Xiao et al 2014). In addition, modulation of neprilysin expression, the most potent Aβ -degrading enzyme (Shirotani et al 2001) may provide an alternative opportunity for intervention (Nalivaeva et al 2012). Neprilysin may act on a wide range of peptide substrates with biological functions. Nevertheless, this problem may be overcome by modifying the neprilysin active site in such a way as to make it more specific to for Aβ degradation (Webster et al 2014). A variety of proteins such as apolipoprotein E and J have been described that interact with Aβ and regulate its ability to across the BBB (Calero et al 2012). Aβ may be degraded in the circulatory system after transport into the blood by either Aβ-degrading enzymes or by immune response cells, such as monocytes that have been shown to clear vascular Aβ (Michaud et al 2013).

1.6.2.1.1 Aβ immunotherapy

Aβ immunotherapy is a potential AD treatment that has been advanced recently because of its ability to decrease brain Aβ accumulation (Frenkel et al 2000). Aβ immunotherapy can be achieved by active immunization, which is performed by injecting AD subjects with synthetic intact synthetic Aβ or synthetic Aβ fragments bound to a carrier protein to mediate an immune response that generate antibodies against Aβ (Bard et al 2000). The antibody bound Aβ may be removed via Fc receptor -mediated clearance by microglia (Bard et al 2000). Aβ immunization results in reduced CNS Aβ load and less neuritic dystrophy, as well as improved cognition

compared to non-treated controls (Bard et al 2000, Masliah et al 2005, Schenk et al 1999, Serrano-Pozo et al 2010). However, active immunotherapy of AD patients was terminated in phase II clinical trials due to an incidence of meningoencephalitis which may have been caused by T cell-induced inflammatory responses (Liu et al 2009, Orgogozo et al 2003).

Passive immunization is another Aβ immunotherapy that is currently being tested in clinical trails. In passive immunization, the antibodies directly against Aβ are administrated to the patients. Using this approach, the antibody may interact with plaques thereby inducing a scavenging response in microglia (Bohrmann et al 2012). Nevertheless, clinical trails using anti-Aβ monoclonal antibodies (bapineuzumab and ponezumab) were discontinued due to the lack of cognitive improvement and to adverse side effects (vasogenic cerebral oedema) (Blennow et al 2012, Burstein et al 2013, Landen et al 2013, Salloway et al 2014). However, a humanized antibody, crenzumab, with the decreased Fc receptor affinity, was safely given at a high dose to patients with a good safety outcome in a phase I trials that has seen the drug moved to phase II trials (Adolfsson et al 2012). Another humanized monoclonal antibody, solanezumab, that recognizes the middle region of Aß and binds soluble monomeric forms of Aß, shows positive effects in mild AD (Lannfelt et al 2014). Nevertheless, phase III trials of solanezumab was failed in mild to moderate AD (Doody et al 2014). This may be because this treatment was administrated too late in the course of AD (Laske 2014).

Taken together, these results indicate that immunotherapy for AD treatment is perhaps one of the most promising areas for future investigation. However, a major challenge for both active and passive immunization may be the delivery of synthetic

Aβ antibodies across the BBB into the CNS (Spencer & Masliah 2014). Besides, Aβ immunotherapy, especially actives immunization in particular has been found to produce persistent autoimmune side effects in an AD mice model. This was manifested by microglial infiltration, which not only activated microglia to engulf Aβ but also importantly led to disruption and degeneration of local tissues (Liu et al 2009). Moreover, it has not been easy to identify any clinical benefits in Aβ immunotherapy in phase II – III clinical trials (Blennow et al 2014). Therefore, active Aβ immunotherapy for AD treatment needs to be more intensively investigated. However, ethics considerations may prohibit further clinical trails. Currently passive immunization strategies demonstrate the feasibility of Aβ clearance, however, more studies will be needed to determine dosage and the stage at which antibodies should be delivered.

1.6.2.2 Stem cell therapy for AD

During the late stage of AD, reduction or elimination of Aβ may not be adequate to overcome the functional loss of neurons and synapses, nor allow for rescue of neuronal dysfunction caused by AD progression (Spencer & Masliah 2014). Therefore, stem cell based therapies have been proposed as a way to repair and replace the deficit or loss of neurons. Stem cell therapeutic strategies could employ two approaches: one is to activate endogenous stem cells, and the other is to transplant regenerated cells or tissues into the damaged or injured sites.

Stem cell transplantation therapy for AD is challenging because many different types of neurons and neurotransmitter systems are affected in AD. In order to incorporate into the complex brain circuit system, stem cells have to migrate into multiple areas of

the brain, differentiate into different types of neuronal cells, and to establish afferent connectivities of physiological relevance with appropriate targets. For this reason, therapeutic strategies based upon cell replacement may not succeed for diffuse neural disorders like AD (Chen & Blurton-Jones 2012). Nonetheless, recent studies indicate that neural stem cell transplantation can improve cognition in AD transgenic mice (Blurton-Jones et al 2009, Yamasaki et al 2007). Thus stem cell therapy has potential for development as a treatment for AD.

Stem cell therapy can also be used to deliver growth factors to the brain. Production of several essential neurotrophins that may regulate synaptic strength and numbers in an activity-dependent manner can be decreased significantly in the early stages of AD, possibly contributing to synapse loss (Arancio & Chao 2007). Neurotrophins, have been reported to improve cognition in patients and they are produced abundantly in NSPCs (Blurton-Jones et al 2009, Kamei et al 2007, Sun et al 2003). Delivery of NSPCs into the AD brain may increase neurotrophin secretion and thereby exert a positive effect on cognition (Tuszynski et al 2005). However, it still unclear whether functional recovery is driven by stem-cell mediated neurotrophin delivery, as lesions or injuries also stimulate production of endogenous neurotrophins (Blurton-Jones et al 2009, Martinez-Serrano & Bjorklund 1996).

Chronic inflammation may play a crucial role in AD neurodegeneration (Akiyama et al 2000). NSPCs can produce anti-inflammatory factors such as interleukin-10 and prostaglandin E2 (Ylostalo et al 2012, Zhou et al 2011a), or can attenuate microgliosis and release of pro-inflammatory cytokine tumour necrosis factor α (TNF α) (Ryu et al 2009). Delivery of anti-inflammatory NSPCs was found to reduce neuroinflammation and result in improved in cognition and in pathology in AD mice (Seo et al 2011).

Evidence showed that anti-inflammatory drugs lower AD risk (Andersen et al 1995, Côté et al 2012), although it may be too late for patients displaying AD symptoms (Aisen 2002). Nevertheless, suppression of the immune system may be a viable therapeutic target for AD, as NSPC transplantation may possibly relieve AD symptoms by decreasing inflammation (Bentham et al 2008, Green et al 2009).

NSPCs may be a promising way to deliver therapeutic proteins to the damaged areas of the brain because NSPCs can migrate throughout brain tissue to the injured and inflamed areas (Muller et al 2006). In addition, some stem cells can stimulate microglia to produce the Aβ degradating enzyme, neprilysin (Kim et al 2012). However, it is still unknown whether survival and differentiation of transplanted stem cells can be affected by AD-related pathology, and whether transplanted cells are influenced by endogenous NSPCs (Chen & Blurton-Jones 2012).

1.7 Biology of neural stem and progenitor cells (NSPCs)

Stem cells, either derived from embryonic or adult CNS, maintain an ability to undergo long-term self- renewal and to differentiation into more highly specialized brain cells. Therefore, stem cells are theoretically considered to be an inexhaustible supply of many cell types for regenerative medicine. Neurological diseases are usually associated with neuronal damage or loss, thus use of stem cells to replace destroyed neural is a potential supplementary therapy for neurological diseases including AD. Moreover, all major types of cells in the CNS are derived from stem cells, thus investigation of stem cell biology may provide new insights into understanding the pathogenesis of neurological disorders.

1.7.1 Embryonic stem cells (ESCs)

Mammalian embryonic stem cells (ESCs) develop from the inner cell mass of the primitive implanted embryo. They are pluripotent and capable of generating the three primary germ layers, ectoderm, mesoderm and endoderm, which will give rise to all types of cells in tissues later in embryogenesis including neural stem cells (Rippon & Bishop 2004). Neural differentiation occurs at an early stage during embryonic development, soon after the specification of the germ layer. Cells of the ectoderm, the outer embryonic germ layer, have the capacity to form both the CNS and the epidermis (Li et al 2013a), while the cells in the margin of ectoderm develop into peripheral nervous system (Marshak et al 2001). The ectoderm is divided into three sets of cells. Internal ectodermal cells form a closed neural tube with a central canal that subsequently develops into the CNS (brain and the spinal cord) (Kalyani et al 1997).

The neural tube is comprised of a single layer of proliferating and morphologically homogenous cells called neuroepithelial cells (Kalyani et al 1997). Neuroepithelial cells can divide symmetrically to generate two identical daughter cells. Alternatively, they can switch to undergo various asymmetrical divisions that produce two distinct daughter cells - a self-renewing neural stem cell and a differentiating neuroblast (Gotz & Huttner 2005). During their transition to neural tissues such as neocortex, early neuroepithelial cells develop into radial glia cells that are mitotically active throughout neurogenesis and undergo both symmetric and asymmetrical cell division (Mission et al 1991). The radial glia cells processes radial morphology with glial characteristics in contrast to epithelial cells (Hartfuss et al 2001, Malatesta et al 2000) and are not only capable of self-renewal, but also participate in the generation of

neurons, astrocytes, oligodendrocytes and ependymocytes during embryonic development (Malatesta et al 2003, Spassky et al 2005). Radial glial cells can serve as a neuronal progenitors in all regions of the CNS during development (Anthony et al 2004) and are present until the end of embryonic neurogenesis and neuronal migration (Noctor et al 2004). The remainder will be transformed into astrocytic adult neural stem/progenitor cells (NSPCs) postnatally residing in the subventricular zone (SVZ) (Merkle et al 2004). However, no matter whether the cells are radial glial or neuroepithelial cells, they are all embryonic neural stem cells.

In the developing CNS, neural stem cell (NSC) proliferation and differentiation are regulated by a number of growth factors. NSC proliferation occurs in response to both epidermal growth factor (EGF) and fibroblast growth factor (FGF) (Tropepe et al 1999). In addition, insulin and insulin – like growth factors (IGFs) are suggested to play a role in embryonic NSC development as insulin has been found to promote NSC proliferation and survival (Desai et al 2011, Freund et al 2008) while IGF-1 immunoreactivity can be detected in the developing brain (Garcia-Segura et al 1991). IGF-1 stimulates NSC proliferation in a way that is distinct from that of EGF and FGF (Arsenijevic et al 2001). Furthermore, IGF - 2 is involved in the modification of cell proliferation and survival in early embryonic stages (Burns & Hassan 2001), and IGF-2 mutant mice display defects in brain development (Baker et al 1993). In addition, brain-derived neurotrophic factor (BDNF) stimulates NSC proliferation (Islam et al 2009) and can promote early radial glial cell differentiation in the developing cerebral cortex by inducing bone morphogenetic protein (BMP) expression in embryonic neurons (Ortega & Alcantara 2010).

1.7.2 Adult neural stem/progenitor cell (NSPCs) and neurogenesis

Neurogenesis, the birth of new neurons, is a process that produces functionally integrated neurons from stem cells or progenitors. This process occurs mostly in the mammalian CNS during embryonesis, but it also occurs in post-natal stages and throughout adulthood in mammals (Altman & Das 1965, Kaplan & Hinds 1977). A variety of studies have revealed that neurons are born, and neural stem/progenitor cell (NSPC) proliferate, during adult life, but only in selected areas of the brain (Alvarez-Buylla & Garcia-Verdugo 2002, Eriksson et al 1998, Reynolds & Weiss 1992, Temple 1989, Temple & Alvarez-Buylla 1999). However, apart from the two classic areas of neurogenesis, the ventricular-subventricular zone (V-SVZ) lining in the lateral ventricle (Alvarez-Buylla & Garcia-Verdugo 2002, Bedard & Parent 2004, Fuentealba et al 2012, Lois & Alvarez-Buylla 1993) and the subgranular zone of the dentate gyrus (DG-SGZ) in the hippocampus (Eriksson et al 1998), other major neurogenic regions may exist (Bordiuk et al 2014) in the adult mouse brain.

Adult neurogenesis in both the V-SVZ and the SGZ is a process that is tightly controlled and regulated. It involves in NSPC maintenance, activation and proliferation, as well as differentiation and migration of the intermediate progenitors to their final destination. Newborn neurons subsequently mature and integrate into the local neuronal network (Hsieh 2012, Taylor et al 2013). The V-SVZ and SGZ in adult brain contain multipotent NSPCs that are capable of self-renewal through cell division and differentiation into neurons and glia (Palmer et al 1999, Reynolds & Weiss 1992, Richards et al 1992). Thus, multipotent NSPCs may be a source of potential precursors useful for transplantation to damaged neural tissues, and thus for the treatment of neurological diseases. Furthermore, alteration of neurogenesis in

neurological injury or disease has been reported (Curtis et al 2003, Gao et al 2009, Hoglinger et al 2004, Jin et al 2004c, Miles & Kernie 2008, Parent et al 1997, Yu et al 2009). Therefore studying adult NSPC proliferation and differentiation may be helpful for understanding how endogenous adult-born neurons may be used for brain repair and restoration, and whether the manipulation of endogenous neurogenesis is feasible for the treatment of neurological disorders.

1.7.2.1 Adult neurogenesis in V-SVZ

Radial glia cells serve as progenitors for many neurons of CNS during embryonic development (Anthony et al 2004). However, they also give rise to adult NSPCs located in the V-SVZ of the lateral ventricle (Merkle et al 2004). The NSPCs in the V-SVZ are located adjacent to the ependymal cell layer, which lines the lateral ventricle separating the ventricular cavity from the V-SVZ (Doetsch et al 1999). In addition, NSPCs also interact with basal lamina projection from the local vasculature (Mirzadeh et al 2008).

Ependymal cells remain quiescent and have no NSPC properties (Doetsch et al 1999). NSPCs contain type B cells expressing astrocyte markers (Doetsch et al 1997, Platel et al 2009) including glial fibrillary acid protein (GFAP) (Bignami & Dahl 1974) and glutamate aspartate transporter (GLAST) (Lehre et al 1995). Expression of GFAP in type B cells is consistent with the idea that radial glia transformed into astrocytic NSPCs in V-SVZ at the end of histogenesis (Merkle et al 2004). Type B cells are relatively quiescent, but they also can exist in an activated state according to recent studies (Codega et al 2014, Mich et al 2014). Quiescent type B cells do not express the intermediate filament protein, nestin (Lendahl et al 1990), which has been thought

of as a NSPC marker (Lim & Alvarez-Buylla 2014). Nevertheless, nestin is expressed in activated type B cells that generate actively proliferating C cells, which serve as neurogenic intermediate progenitor cells (nIPC) or transiting amplified progenitors in the V-SVZ of adult brain (Doetsch et al 1999). Type C cells divide symmetrically or asymmetrically and are able to generate neuroblasts (type A cells) that migrate to the olfactory bulb through (RMS) (Fig. 1.3) (Belluzzi et al 2003, Lois & Alvarez-Buylla 1994). Type C and A cells are also labelled by specific molecular markers. Despite expression of the immature neuronal marker doublecortin (DCX), type A cells are positively stained by anti-PSA-NCAM (Rousselot et al 1995, Rousselot & Nottebohm 1995) and anti-TuJ1 antibodies (Doetsch et al 1997). PSA-NCAM is a protein expressed by migrating neuroblasts (Doetsch & Alvarez-Buylla 1996, Rousselot et al 1995) while the TuJ1 antibody labels β III tubulin in young neurons (Easter et al 1993, Moody et al 1996). However, ependymal cells, type B and type C cells do not express PSA-NACM and β III tubulin (Doetsch et al 1997), whereas type A and C cells strongly express GFAP. Both type A and type C cell types are immunoreactive with nestin antibodies (Doetsch et al 1997).

Apart from producing type A cells, type B and C cells may contribute to the generation of oligodendrocytes (Menn et al 2006). NG2-expressing cells that are known as oligodendrocyte progenitors (OPCs) show a type C cell - like immunophenotype during early postnatal and adult stages in the V-SVZ (Aguirre et al 2004). In addition, a small sub-population of type C cells in the V-SVZ express oligodendrocyte lineage transcription factor 2 (Olig2) (Menn et al 2006) that guides astrocyte and oligodendrocyte formation in the V-SVZ (Marshall et al 2005). Furthermore, Olig2 - expressing type B cells can give rise to a small number of non-

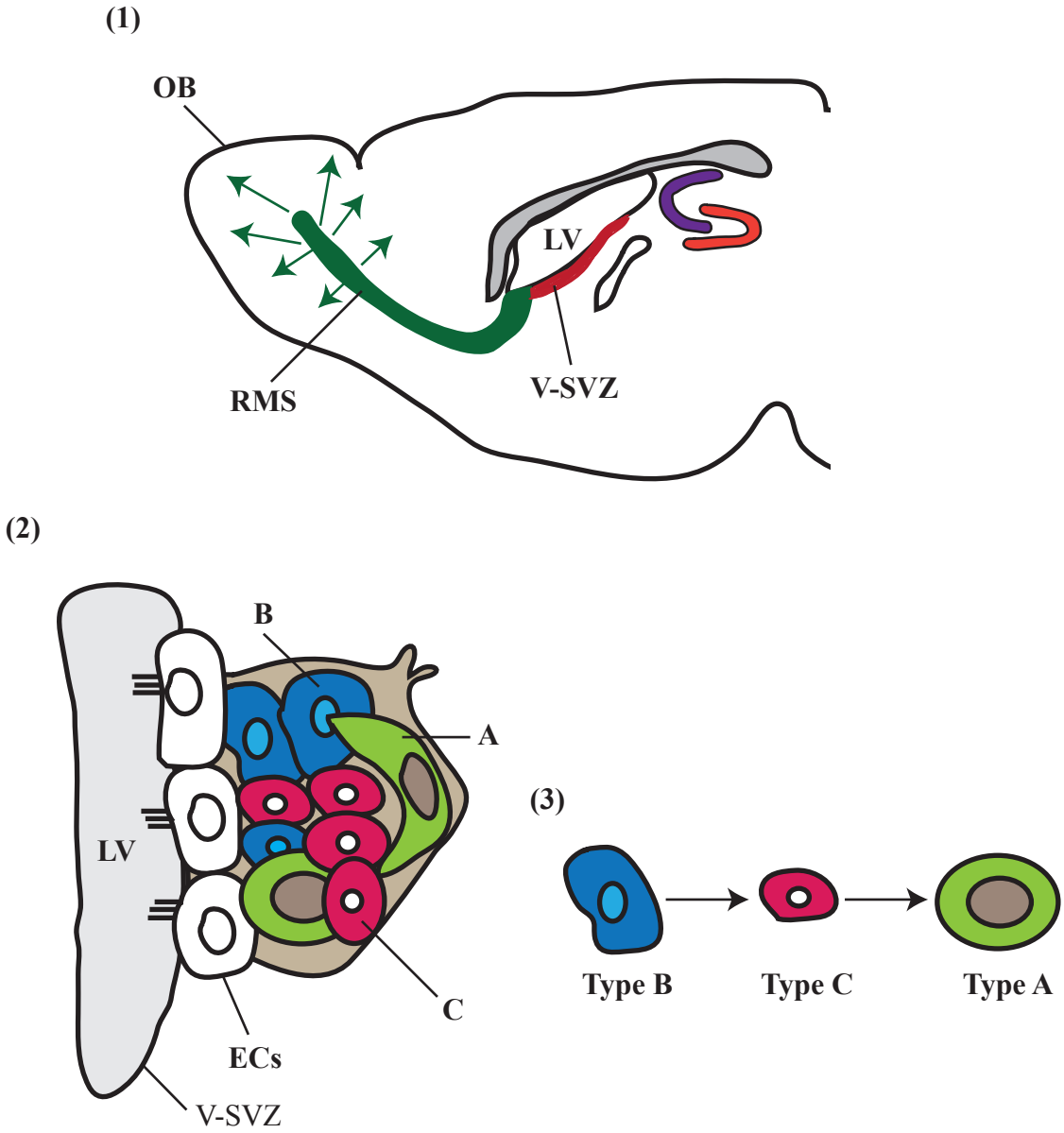

Figure 1.3 Schematic illustration of adult neurogenesis in the adult ventricle - subventricular zone (V-SVZ). (1) The V-SVZ is located in the wall of the lateral ventricles. (2) The V - SVZ contains ependymal cells (ECs, white), comparatively quiescent type B cells (blue), transit-amplifying type C cells (red) and neuroblasts type A cells (green). (3) In the V-SVZ, GFAP-positive astrocytes (type B cells) act as neural stem/progenitor cells that are able to generate nestin positive transit-amplifying cells (type C cells) which differentiate into neuroblasts (type A cells). Neuroblasts generated in the V-SVZ migrate towards the olfactory bulb (OB) along the rostral migration stream (RMS).

myelinating NG2 expressing cells and myelinating mature oligodendrocytes (Menn et al 2006). Moreover, enhanced generation of new oligodendrocytes from NSPCs in SVZ migrating to injury sites can be detected in vivo after demyelinating lesions of the corpus callosum (Picard-Riera et al 2002). Therefore, the NSPCs in the SVZ may generate new oligodendrocytes under both normal and injury conditions in the adult brain (Menn et al 2006).

Neuroblasts (type A cells) originating from NSPCs in the SVZ migrate through the RMS for a distance up to 5 millimeters in the adult mouse brain (Doetsch & Alvarez-Buylla 1996). Neuroblasts undergo tangential directional migration to the olfactory bulb (OB) via a chain migratory manner to form elongated cell aggregates that are ensheathed by astrocytes (type B cells) (Lois et al., 1996). This directional chain migration of neurobasts along the RMS is regulated by a number of factors (Belvindrah et al 2007, Cao et al 2013, O'Leary et al 2014, Platel et al 2009, Young et al 2010). For instance, neuroblasts survival and migration are controlled by SVZ astrocytes via glutamate signaling through NMDARs (Platel et al 2010). In the RMS, PSA-NCAM expressing cells (type A cells) are more abundant than GFAP-expressing type B cells, but no type C cells could be detected (Doetsch et al 1997). Most neuroblasts (type A cells) migrate through the RMS into the OB where they differentiate into interneurons (Lois et al 1996, Whitman & Greer 2007a). Beside the RMS, neuroblasts also migrate to populate the cortex and subcortex regions in the first few postnatal weeks (De Marchis et al 2004, Sanai et al 2011). In addition, in - vivo studies suggest that glia migration may not follow the classical RMS route (Menn et al 2006, Picard-Riera et al 2002, Seri et al 2006). Moreover, SVZ - derived neuroblasts migrate toward lesion sites where they differentiate into mature neurons

(Kernie & Parent 2010, Sundholm-Peters et al 2005, Yamashita et al 2006). This highlights the fact that simulating neurogenesis in the SVZ may be a potential therapeutic strategy for neurological disease.

Upon reaching the center of the OB, neuroblasts detach from the chain, and turn radically and move toward the granular cell layer, and then to the periglomerular cell layer (Lois et al 1996). Upon arrival at the destination, neuroblasts start to differentiate into interneurons. Specification of OB interneurons has genetic (Kohwi et al 2005, Saino-Saito et al 2007, Waclaw et al 2006) spatial and temporal (Lledo et al 2006) contributory factors. NSPCs from the V-SVZ give rise to two main types of interneurons in the OB. One type reaches the granular cell layer where the cells differentiate into granular neurons, whereas the other moves into the periglomerular layer and develops into periglomerular neurons (Petreanu & Alvarez-Buylla 2002, Yang 2008). To integrate and survive, the newly generated neurons receive either GABAergic or glutamatergic synaptic inputs from surrounding neurons and axonal terminals (Eyre et al 2008, Laaris et al 2007).

Granule cells (GCs) comprise the majority of the OB. Most of the GCs in the OB are produced postnatally and are added constantly in adulthood (Bayer 1983, Kaplan & Hinds 1977). Petreanu & Alvaren-Buylla (2002) first described the maturation of GCs generated from adult. After arriving at the granule cell layer, newborn axonless GCs soon project basal and apical dentrites to form synaptic connections and functionally integrate with existing OB circuitry (Belluzzi et al 2003, Petreanu & Alvarez-Buylla 2002). However, the survival selection and synaptic development of adult generated GCs are determined by the level of activity they receive (Balu et al 2007, Whitman &

Greer 2007b) e.g. GABAergic (Eyre et al 2008, Pallotto et al 2012), glutamatergic (Laaris et al 2007), noradrenergic (Veyrac et al 2005) and cholinergic (Devore & Linster 2012, Kaneko et al 2006, Pressler et al 2007) stimulation occurring in critical time windows (Ge et al 2007, Kelsch et al 2009) when neurons have a high degree of plasticity (Petreanu & Alvarez-Buylla 2002, Yamaguchi & Mori 2005). Almost 50% of the GCs die after one month, and the rest are durably integrated into the OB (Petreanu & Alvarez-Buylla 2002, Winner et al 2002). Only less than 10% of newborn GCs survive more than 21 months. Nevertheless, the dead or older cells will be replaced by newborn GCs (Kaplan et al 1985).

Compared to GCs, newborn interneurons differentiate into periglomerular cells (PGCs) more slowly, with a delay of nearly one month. PGCs receive either GABAergic or glutamatergic input from olfactory sensory axons and from the apical dentrites of principal neurons in the OB (Grubb et al 2008). Survival and synaptogenesis of PGCs also process in a sensory activity - dependent manner (Grubb et al 2008, Mandairon et al 2006). PGCs generally have axons, but axonless types may also exist (Kosaka & Kosaka 2011). Three subtypes of adult - born PGCs have been described based upon their immunoreactivity to tyrosine hydroxylase (TH), an enzyme required for dopamine synthesis, to the calcium - binding proteins calbindin (CalB) and calretinin (Parrish-Aungst et al 2007). However, PGCs can also be grouped into two types, depending upon whether they do or do not receive synapses from olfactory nerves (Kosaka et al 2001, Kosaka & Kosaka 2011). Compared to GCs, the newly generated PGCs display a similar decline in cell numbers during maturation, with the remaining PGCs survive up to 19 months (Winner et al 2002).

Although NSC proliferation and differentiation occur persistently in the V-SVZ-OB region (Lois & Alvarez-Buylla 1993, Morshead et al 1994), the volume of the OB does not seem to increase (Pomeroy et al 1990). Apoptosis could be detected in the layer into which the newly produced neurons flow. This not only maintains the OB structure but also essentially contributes to neural plasticity of olfactory information processing (Fiske & Brunjes 2001, Imayoshi et al 2008, Lepousez et al 2013, Sakamoto et al 2014).

Adult neurogenesis in the V-SVZ-OB system is affected and regulated by a number of factors. Type B cell bodies extend short processes through the ependymal layer via apical endings that directly contact cerebrospinal fluid (CSF). The CFS contains multiple soluble factors (eg neutrophins, growth factors) that may regulate NSPC maintenance and behaviour (Mirzadeh et al 2008, Shen et al 2008). Several extrinsic signals including bone morphogenetic proteins BMPs, sonic hedgehog (Shh), and Wingless related integration site (Wnt) proteins are present in CSF and modulate neurogenesis in V-SVZ (Huang et al 2010, Lehtinen et al 2011). Ependymal cells growing a large number of long motile cilia into the lateral ventricle, and recent studies report that primary cilia are crucial for Shh signal transduction (Huangfu & Anderson 2005, Lehtinen et al 2011). In addition, blood vessels in the V-SVZ zone contain a substantial extracellular matrix (ECM) that is closely associated with type B and C cells (Shen et al 2008, Tavazoie et al 2008), and endothelial derived factors may also play an important role in NSPC proliferation and neuroblast production (Katsimpardi et al 2014, Shen et al 2004). Furthermore, V-SVZ neurogenesis is also affected by hormones (Lau et al 2007, Lopez-Juarez et al 2012) and different types of neurotransmitters especially at stages of integration and maturation, such as GABA

(Alfonso et al 2012, Liu et al 2005), dopamine (Kim et al 2010, O'Keeffe et al 2009) and acetylcholine (Paez-Gonzalez et al 2014). Additionally, multiple transcriptional factors participate in the modulation of NSPC commitment and fate specification. SOX2, a sequence - specific DNA - binding transcription factor exerts effects in many essential processes of adult NSPC (Sarkar & Hochedlinger 2013, Shimozaki et al 2012). Sox 2 cooperates with TLX transcription factors, and thereby mediate type B cell proliferation (Ellis et al 2004, Shimozaki et al 2012). Expression of basic helix - loop - helix (bHLH) transcritption factor-neurogenin 2 (Ngn2), found in some type C cells, induces generation of calretinin – positive OB neurons (Roybon et al 2009b). Interestingly, microglia are suggested to promote generation of neurons and oligodendrocytes in V-SVZ by the release of cytokines (Gonzalez-Perez et al 2010, Shigemoto-Mogami et al 2014).

1.7.2.2 Adult neurogenesis in subgranular zone of dentate gyrus (DG-SGZ)

The subgranular zone (SGZ) of the hippocampus is another major region that generates new neurons in adult mammalian brain (Altman & Das 1965, Eriksson et al 1998, Kaplan & Hinds 1977). The SGZ zone, where hippocampal neurons are born, is located at the interface of the dentate gyrus granular cell layer and the hippocampal hilus (Seri & Alvarez-Buylla 2002). Two types of dividing NSPCs, radial astrocytes and small cells with basophilic nucleic that stained darkly with hematoxyin, have been found in the SGZ (Altman & Das 1965, Cameron et al 1993, Kaplan & Hinds 1977, Palmer et al 2000). Classic anatomical studies suggest that adult radial astrocytes in the SGZ are derived from radial glial cells in the embryo (Altman & Bayer 1990, Eckenhoff & Rakic 1984).

Proliferating radial astrocytes and other precursors generate intermediate precursors, which in turn give rise to neuroblasts in the SGZ. The neuroblasts are generated from immature neurons that migrate to the granular cell layer and continuously differentiate into granule cells (Kempermann et al 2003, Kuhn et al 1996, Seri & Alvarez-Buylla 2002). The putative NSPC in the SGZ is a type of radial glia - like cell that expresses both the astrocyte maker GFAP and the neural precursor marker nestin. This cell is known as the type I cell (Filippov et al 2003, Seri et al 2001). In addition to acting as a neuronal precursors, type I cells also share astrocytic electrophysiology properties, and retain astrocyte features, such as the ability to interact with the vasculature (Filippov et al 2003, Fukuda et al 2003). Factors coming from blood vessels may affect NSPC behavior in the DG-SGZ (Palmer et al 2000). Type I cells, like GFAP expressing - type B cells in the SVZ, represent only 5% of the nestin-expressing cell population in SGZ (Filippov et al 2003, Kronenberg et al 2003, Lugert et al 2010). The slowly dividing quiescent type I cells do not generate neurons directly, but give rise to dark staining small cells, termed type II cells (Filippov et al 2003, Fukuda et al 2003).

Type II cells, have short separate processes and dense - shaped nuclei. They do not express GFAP and are highly amplified cells (Filippov et al 2003, Kempermann et al 2004, Kronenberg et al 2003). Type II cells are nestin - positive and are defined as transit amplified neurogenic intermediate progenitors. Type II cells can be classified into two subtypes depending on whether they express DCX or not (Brown et al 2003). Transit amplified type II cells can potentially generate astrocytes or neurons in the SGZ (Encinas et al 2011, Kempermann et al 2004, Kronenberg et al 2003). Type III cells are nestin - negative and DCX - positive, but still have the capacity to

proliferate. They express more neuronal features than type II cells. Type III cells are also referred as neuroblasts (Seaberg & van der Kooy 2003, Weissman et al 2001). Up – regulation of the interneuron marker calretinin and neuronal marker NeuN as well as down - regulation of DCX occurs during differentiation and development of immature neurons into mature glutamatergic granule neurons (Hsieh 2012).

Unlike migration in SVZ, newborn neurons only migrate a short distance in the SGZ before arriving at the granular cell layer. The newly generated granule cells in the dentate gyrus exhibit similar electrophysiology characteristics to mature granule cells, but they continually undergo morphological and physiological alterations (Ge et al 2007, Toni et al 2007), as they grow dendrites to the molecular layer and extend axons to the CA3 region through the hilus (Zhao et al 2006). The newly produced granule cells integrate into the existing circuitry, and are initially activated by ambient GABA produced by surrounding interneurons (Bhattacharyya et al 2008, Ge et al 2006). They subsequently receive GABAergic synaptic inputs and then glutamatergic synaptic inputs (Esposito et al 2005, Ge et al 2006, Schmidt-Salzmann et al 2014), and finally they form synaptic contacts with hilar and CA3 neurons through mossy fiber output (Faulkner et al 2008, Toni et al 2008). However, adult - generated neurons display hyperexcitability and increased synaptic plasticity during development at specific stages compared to the fully mature granular neurons (Ge et al 2008, Schmidt-Hieber et al 2004). Importantly, newborn neurons have to receive GABAergic synaptic and glutamatergic inputs in order to survive (Laplagne et al 2006, Laplagne et al 2007).

Adult neurogenesis in the DG-SGZ is also regulated by multiple physiological, environmental and pathological factors including exercise (Kempermann et al 1998) and neurological disorders (Monje et al 2003, Parent et al 1997). Micro-environmental signalling from cellular compartments such as vascular cells, glia cells as well as granular neurons (Ma et al 2005, Palmer et al 2000, Tavazoie et al 2008, Tozuka et al 2005), and from extracellular matrix (Wojcik et al 2009, Wojcik-Stanaszek et al 2011), exert effects on NSPC activity in the SGZ. In addition, growth factors (Cheng et al 2002), including neurotrophins (Colditz et al 2010), as well as cytokines (Cheng et al 2002) and hormones (Cameron et al 1998) also contribute to the regulation of adult neurogenesis in the DG-SGZ. The neurotransmitters, glutamate (Esposito et al 2005) and GABA (Ge et al 2006), acetylcholine are also suggested to directly modify migration, maturation, integration and survival of newborn neurons in the hippocampus (Cooper-Kuhn et al 2004, Vivar et al 2012). In addition, cell to cell signalling by Notch (Ables et al 2010), Shh (Ahn & Joyner 2005), BMP (Mira et al 2010) and Wnt (Lie et al 2005) potentially participate in hippocampal neurogenesis regulation. This may be achieved through a series of transcriptional factors, for example the transcriptional factor Sox2 (Lefebvre et al 2007) is involved in NSPC self renewal, and requires active Notch signalling to maintain expression in NSPCs (Ables et al 2010, Ehm et al 2010, Imayoshi et al 2010). Furthermore, a number of transcriptional factors are also reported to control neurogenesis at different stages (Hodge et al 2008, Lugert et al 2010, Roybon et al 2010). For instance, the bHLH transcriptional factor Hes5 was found to colocalize with Sox2 and thereby collaborate to regulate NSPC maintenance (Lugert et al 2010), whereas another bHLH-transcriptional factor Ngn2, is up-regulated expressing only during transition from type I cell to neuroblasts, and is suggested to be crucial for specifying neuronal fate

during hippocampal neurogenesis (Roybon et al 2009b). Microglia also play a role in the regulation of adult neurogenesis by phagocytosis of newborn neurons undergoing apoptosis (Sierra et al 2010).

1.7.2.3 Function of adult neurogenesis

The generation of new neurons from adult NSPCs in the DG-SGZ, V-SVZ and other neurogenic niches in the adult brain has implicated in learning (Dupret et al 2007) and memory (Akers et al 2014), mood regulation (Snyder & Cameron 2012, Snyder et al 2011) as well as in neural repair following brain dysfunction or damage (Lazarini et al 2014, Sun 2014). In this section, the role of neurogenesis in regulating these functions is reviewed.

Newly generated neurons are thought to participate in olfactory- and hippocampal-dependent learning and memory (Braun & Jessberger 2014, Moreno et al 2009). New neurons derived from V-SVZ can interconnect with olfactory circuits and contribute to circuit maintenance and olfaction (Cummings et al 2014). This integration triggers learned behaviour responses and thereby promotes long-term memory (Lepousez et al 2013, Moreno et al 2009, Scotto-Lomassese et al 2003, Sultan et al 2010). Ablation of newborn neurons in the OB specifically impairs odour associative long-term memory (Arruda-Carvalho et al 2011). Interestingly, either odour associative or non-associative odour learning has been proposed to promote survival of newly incorporated neurons in OB (Belnoue et al 2011, Lepousez et al 2014), which implies that V-SVZ olfactory neurogenesis may not only contribute to, but may also be regulated by learning and memory.

Newly generated neurons derived from adult hippocampal neurogenesis are also suggested to be involved in learning acquisition and long term - memory formation (Suarez-Pereira et al 2014). Disruption of adult neurogenesis in hippocampus by radiation treatment impairs contextual learning and memory formation (Hernandez-Rabaza et al 2009, Kee et al 2007, Stone et al 2011, Winocur et al 2006). However, other results suggest that the link between adult hippocampal neurogenesis and learning and memory may not be so clear (Groves et al 2013, Luu et al 2012, Saxe et al 2006). These inconsistences may be caused by multiple variables such as incomplete block to neurogenesis, unexpected off-target effects and different tasks applied to test learning and memory abilities (Deng et al 2010, Hernandez-Rabaza et al 2009).

Adult neurogenesis is also suggested to influence mood, as stress-induced down-regulation of hippocampal neurogenesis has been postulated to contribute to depression (Lehmann et al 2013, Snyder et al 2011, Surget et al 2011). The hippocampus plays a role in modulating stress via the hypothalamic pituitary adrenal (HPA) axis by controlling the release of cortisol from the adrenal gland (Herman et al 1989, Mizoguchi et al 2003). Several studies show that reduced hippocampal neurogenesis occurs under conditions of increased cortisol levels or chronic stress (Gould et al 1998, Lagace et al 2010, Lehmann et al 2013, Murray et al 2008). Disrupted hippocampal neurogenesis also causes higher levels of anxiety as well as a longer lasting elevation in blood cortisol levels. This suggests that newly generated neurons may regulate the HPA axis via a feedback loop following stress, and that this may in turn regulate cortisol levels which affect neurogenesis (Snyder et al 2011, Surget et al 2011). In addition, several studies show increased numbers of adult

hippocampal NSPCs following chronic treatment with clinical antidepressants (Boldrini et al 2009, Duman 2004, Malberg et al 2000, Perera et al 2007), which supports the idea of a link between adult neurogenesis and depression. However, the relationship between neurogenesis and depressive behavior is also questioned by various studies showing that ablation of adult neurogenesis does not affect anxiety or depressive-like behaviors (Jayatissa et al 2009, Santarelli et al 2003, Wang et al 2008). The conflicting results from these groups may be attributed to inadequate inhibition of neurogenesis or different methodologies used to monitor anxiety or depression.

In addition the relief from depressive behaviour may not be due to enhanced neurogenesis, although exposure to antidepressant treatments has been reported to promote neurogenesis (Malberg et al 2000). For example, an antidepressant agent, fluoxetine was reported to stimulate NSPC proliferation (Malberg et al 2000). Nevertheless, fluoxetine can relieve depression by increasing synaptic serotonin availability through its action as a serotonin reuptake inhibitor (Rahmani et al 2013, Ranganathan et al 2001). Interestingly, serotonin was observed to promote granule cell proliferation (Brezun & Daszuta 2000, Rahmani et al 2013). Thus, inhibition of serotonin reuptake may increase NSPC proliferation. More importantly, no change has been reported in NSPC proliferation in subjects with depressive like symptoms (Reif et al 2006). Therefore, the potential role of adult neurogenesis in mood regulation including its role in clinical depression, needs further clarification.

In general, enhanced adult neurogenesis occurs in a number of pathological conditions such as during seizure (Parent et al 1997), schizophrenia (Reif et al 2006)

or traumatic brain injury (Gao et al 2009). However, increased adult neuron generation may not always contribute to symptom relief. Indeed, it may even make the pathological situation worse in some cases. For example, seizures induce increased number of dentate granule cells in the adult rat hippocampus, and these new granule cells are committed to abnormal network reorganization (Parent et al 1997). This may negatively affect local existing circuit in hippocampus. Nevertheless, some neural damage may benefit from enhanced adult neurogenesis. For instance, increasing neurogenesis via physical activity seems to improve behavioural deficits in a mouse model of schizophrenia (Wolf et al 2011). Therefore, the role of adult neurogenesis in generating functional neurons for repair and replacement of the damaged or dysfunctional cells needs to be clarified in specific neurological disorders.

1.7.2.4 Factors influencing NSPC proliferation and differentiation

To produce new functional neurons, astrocytes or oligodendrocytes, a NSPC must undergo proliferation, migration and differentiation and then integrate into existing cellular networks (Christie & Turnley 2012). The NSPC life cycle and biology in the adult brain under both normal or pathological conditions is tightly regulated by multiple factors including a variety of cell-signalling pathways (Huangfu & Anderson 2005, Lie et al 2005), growth factors (Cheng et al 2002, Teramoto et al 2003), cytokines and chemokines (Bajetto et al 2002, Turbic et al 2011), as well as extracellular matrix (ECM) molecules (Wojcik-Stanaszek et al 2011). In particular, proliferation and differentiation may be modified by these factors to promote cell production and the generation of specific neural cell types after CNS injury (Lu et al

2011, Schabitz et al 2007, Zhu et al 2011). In this section, several factors affecting cell proliferation and differentiation are reviewed.

1.7.2.4.1 Growth factors

Various growth factors including epidermal growth factor (EGF) (Ayuso-Sacido et al 2010), fibroblast growth factor (FGF) (Palmer et al 1999), nerve growth factor (NGF) (Scardigli et al 2014), brain derived neurotrophic factor (BDNF) (Ahmed et al 1995), glial cell line-derived neurotrophic factor (GDNF) (Boku et al 2013, Heuckeroth et al 1998), insulin-like growth factor 1 (IGF-1) (Arsenijevic et al 2001), and vascular endothelial growth factor (VEGF) (Calvo et al 2011) participate in the modulation of NSPC proliferation and differentiation. Increased expression of these growth factors has been found following ischemia and traumatic brain injury (Christie & Turnley 2012).

1.7.2.4.1.1 Epidermal growth factor (EGF)

EGF, a 5.5 kD peptide, is mitogen that is widely used to stimulate NSPC proliferation and to maintain NSPC growth in cell culture (Reynolds & Weiss 1992). EGF is a ligand that binds to the EGF receptor (EGFR) that is expressed by both type B and type C cells in the V-SVZ (Doetsch et al 2002) as well as by radial astrocytes in the SGZ (Jin et al 2002). Activation of EGFR tyrosine kinase activity promotes NSPC proliferation, differentiation, migration and survival (Aguirre et al 2010, Gonzalez-Perez & Quinones-Hinojosa 2010, Gonzalez-Perez et al 2009). Transit amplified NSPCs (type C cells) in V-SVZ express high levels of EGFR, and can be induced by EGF to form a multi-potent state capable of producing either neuronal or glial precursors (Doetsch et al 2002). The generation of EGF in the V-SVZ seems to be

essential for maintenance of the proliferative NSPC pool (Christie & Turnley 2012), while EGF infusion into the SGZ appears to promote NSPC proliferation in response to brain injury (Sun et al 2010). Interestingly, infusion of EGF into the uninjured mouse brain also increases NSPC number (Cooke et al 2011) but promotes significantly more glial differentiation than neuronal differentiation (Craig et al 1996, Kuhn et al 1997). However, EGF induces a different extent of NSPC glial differentiation and especially oligodendrocyte generation and remyelination upon brain damage (Aguirre et al 2007, Cantarella et al 2008, Gonzalez-Perez et al 2009), although there are reports that disagree with this opinion (Craig et al 1996, Gonzalez-Perez et al 2009, Kuhn et al 1997).

The precise cellular and molecular basis for the EGF effect on NSPC maintenance and differentiation is not clear (Christie & Turnley 2012, Nieto-Estevez et al 2013). However, EGF is suggested to promote NSPC proliferation and possibly to induce glial differentiation through activation of EGFR (Kuhn et al 1997, Lindberg et al 2012). Several other ligands of EGFR such as β-cellulin (Gomez-Gaviro et al 2012) and transforming growth factor alpha (TGF-α) (Fallon et al 2000, Tropepe et al 1997) have also been proposed to increase NSPC growth through activation of the EGFR (Schneider et al 2008). In addition, inhibition of EGFR activity is reported to promote neuronal differentiation in a spinal cord injury model (Ayuso-Sacido et al 2010, Ju et al 2012, Li et al 2013b). Therefore, EGF may bind to and activate EGFR associated signaling pathway to affect NSPC proliferation and differentiation.

1.7.2.4.1.2 Fibroblast growth factor-2 (FGF-2)

FGF-2 is another mitogen hat is usually combined with EGF to stimulate NSPC proliferation in culture (Reynolds & Weiss 1992). FGF-2 has a molecular weight of 18 kDa. Nonetheless a 24 kDa isoform has also been reported (Powers et al 2000). FGF-2 is mainly produced and secreted by glial cells, particularly by mature astrocytes in the CNS (Reuss et al 1998). FGF-2 regulates NSPC activity in the neurogenic niche by acting on FGF receptors (FGFR) (Galvez-Contreras et al 2012). Generally, four types of FGFRs are expressed differently in different neurogenic locations in the adult brain, with FGFR-1 and 2 occurring in dividing V-SVZ NSPCs, and FGFR-3 only in non-proliferative progenitors in the V-SVZ (Frinchi et al 2008). Notably, FGFR-1 is likely only expressed by NSPC in the SVZ of lateral ventricle (Frinchi et al 2008).

Exogenous infusion of FGF-2 into the lateral ventricle stimulates NSPC proliferation in V-SVZ under normal physiological conditions (Kuhn et al 1997), while inhibition of the release of FGF-2 or pharmacological block of the FGF receptor (FGFR) does not affect NSPC neuronal differentiation. This suggests an important role for FGF-2 in mitotic activity of NSPC in the V-SVZ, but not in the induction of neuronal fate (Agasse et al 2007). FGF-2 knockout mice do not show a significant reduction in NSPC numbers in hippocampus, but display decreased hippocampal NSPC neuronal differentiation after brain injury (Werner et al 2011), which implies that the endogenous production of FGF-2, or activation of FGFR-1, is important for stimulation of NSPC growth and development in the hippocampus following brain damage (Werner et al 2011, Yoshimura et al 2001). However, brain insult promotes up-regulation and accumulation of FGF-2 in NSPCs, which efficiently migrate to the

injured cortex and produce an enhanced pool of immature neurons available for brain repair (Dayer et al 2007, Ganat et al 2002, Wagner et al 1999). Furthermore, FGF-2 supplement in cell culture medium seems to indirectly induce NSPCs to differentiate into astrocytes (Song & Ghosh 2004), and EGF-2 also appears to promote astrocyte activation after brain injury (Reuss et al 1998). Moreover, FGF2 regulates oligodendrocyte progenitors (OPCs) repair process in response to demyelination (Dayer et al 2007, Dehghan et al 2012, Frost et al 2003), as up-regulation of FGF2 ligands and FGFR expression are found in remyelinating lesions (Messersmith et al 2000). Several studies reported similar results i.e. that infusion of EGF in combination with FGF-2, generally improves neural repair through promotion of V-SVZ - derived NSPC proliferation and survival, and repopulation of neurons at the injured areas, leading to functional recovery (Nakatomi et al 2002, Oya et al 2008, Tureyen et al 2005, Winner et al 2008). However, these results do not agree with all published studies (Baldauf & Reymann 2005). Thus the function of FGF-2 in NSPC behaviour has not been fully elucidated. Nevertheless, FGF-2 gene transfer rescue of hippocampal functions in AD brain (Kiyota et al 2011) may be a therapeutic approach for neurodegenerative diseases via manipulation of NSPC growth factors.

1.7.2.4.1.3 Insulin and insulin-like growth factors (IGFs)

EGF and FGF-2 probably are the most potent mitogens for NSPC proliferation, but other growth factors like insulin are also found to stimulate cell proliferation (Rafalski & Brunet 2011), as can IGFs (IGF-1 and IGF-2) (Arsenijevic et al 2001). As a hormone that assists cell store glucose and fats as energy sources (Kadowaki et al 2003, Terauchi & Kadowaki 2002), insulin is also essential for brain function and maintenance (McNay 2007, Rafalski & Brunet 2011, Zhu et al 1990). While it is not

clear how insulin affects NSPC fate, withdrawal of insulin from the cell culture medium leads to autophagic death of NSPCs (Yu et al 2008), whereas high concentration of insulin induce neuronal differentiation of postnatal NSPCs (Han et al 2008). In general, the standard culture media for mouse, rodent and human NSPCs contain insulin and are required for NSPC survival in vitro (Rafalski & Brunet 2011, Yu et al 2008).

Insulin is produced from β cells of the pancreas and enters the brain across the blood brain barrier (BBB) in a receptor-mediated manner (Banks et al 1997a, Banks et al 1997b, Schwartz et al 1991). IGFs are synthesized and released by the liver into blood circulation. Like insulin, IGFs can be transported across the BBB (Reinhardt & Bondy 1994). Furthermore, IGF-1 is also produced in the adult brain, especially in regions of cells undergoing division such as the hippocampus, the V-SVZ and the OB (Bartlett et al 1991). This suggests that IGFs have an important role in NSPC proliferation (D'Ercole et al 1996).

Insulin and IGFs receptors are expressed in adult NSPC, and a number of studies have raised the possibility that insulin, IGF-1, and IGF-2 supply instructive signals to regulate the fate of NSPCs through activation of their corresponding receptors (Bracko et al 2012, Hsieh et al 2004, Supeno et al 2013). For example, insulin is suggested to promote proliferation and prolong survival of NSPCs by binding to insulin receptors which are expressed by NSPCs in culture (Erickson et al 2008). In addition, IGF-1 has been implicated in the control of long - term EGF/FGF2-responsive NSPC proliferation, and in the neuronal differentiation of NSPCs

(Kouroupi et al 2010, Supeno et al 2013, Zhang et al 2014). Furthermore, IGF-2 has also been proposed to regulate postnatal neurogenesis (Bracko et al 2012), as IGF-2 exerts its effects on stem cell differentiation through binding to insulin (Ziegler et al 2014).

1.7.2.4.2 Other factors affecting NSPC proliferation and differentiation

Apart from growth factors, NSPC proliferation or differentiation is regulated by a series of factors such as neurotrophins (Ahmed et al 1995, Scardigli et al 2014), morphogens (Ahn & Joyner 2005, Lie et al 2005, Lim et al 2000), cytokines and chemokines (Bajetto et al 2002, Barbero et al 2002) and transcription factors (Ahmed et al 2009). Notably, a few studies indicate that cysteine protease inhibitors may also participate in regulation of NSPC behavior (Hong et al 2002, Santos et al 2012). FGF induced NSPC proliferation was reported to require a cysteine protease inhibitor, CysC (Taupin et al 2000).

The role of neurotrophins is described briefly in this literature review because neurotrophins have also been reported to affect NSPC proliferation and differentiation. In the course of my studies, it was necessary to consider all potential factors that could mediate the effect of APP on NSPC proliferation and differentiation. Binding of nerve growth factors (NGF) to TrkA receptors and to a much lesser extent the binding of brain - derived neurotrophic factor (BDNF) to TrkB receptors have been suggested to exert effects on differentiation and proliferation of NSPCs (Ahmed et al 1995, Frielingsdorf et al 2007, Islam et al 2009, Scardigli et al 2014). However, the opposite effect of BDNF on NSPC behaviour has also been reported. BDNF did not stimulate neurogenesis in SVZ (Galvao et al 2008).

The morphogens, Wnt (Adachi et al 2007, Lie et al 2005), Shh (Ahn & Joyner 2005, Huangfu & Anderson 2005) and BMPs (Lim et al 2000) are also suggested to regulate NSPC proliferation and maintenance. For example, Wnt signalling increases NSPC proliferation in the neurogenic areas (Adachi et al 2007, Lie et al 2005). Shh, a soluble protein that is normally produced by astrocytes in the neurogenic niche of the adult brain is suggested to regulate NSPC proliferation both in vitro and vivo (Lai et al 2003, Ruiz i Altaba et al 2002). Finally, BMP signalling has been reported to suppresses neuronal and oligodendroglial differentiation of NSPC, but induce astrogliogenesis of NSPC during adult life (Lim et al 2000). Nevertheless, BMP inhibitor expression in the V-SVZ or SGZ can block the BMP signalling and thereby increases neurogenesis (Bonaguidi et al 2008, Lim et al 2000).

Chemokines also have been suggested to play a role in the regulation of NSPC proliferation, differentiation migration and survival (Bajetto et al 2002, Barbero et al 2002, Cartier et al 2005). Adult NSPCs can express chemokine receptors (CCRs) and chemokines that are basally produced in the CNS, especially in the neurogenic regions (Tran et al 2004, Turbic et al 2011). Specific chemokines have been shown to stimulate NSPC proliferation (Filippo et al 2013, Tran et al 2004, Turbic et al 2011). For instance, the pro-inflammatory cytokines interferon gamma (IFγ) and TNFα are major regulators of CCRs and chemokines in the CNS and in other tissues (Hiroi & Ohmori 2003, Suyama et al 2005). Although IFγ and TNFα inhibit NSPC proliferation (Ben-Hur et al 2003, Iosif et al 2006), they can induce neuronal differentiation as well (Ben-Hur et al 2003, Kajiwara et al 2005, Lum et al 2009, Wong et al 2004). There are additionally several other cytokines that are suggested to regulate NSPC proliferation and differentiation (Ernst & Jenkins 2004, Heinrich et al 2003, Kamimura et al 2003, Lan et al 2012, Turnley & Bartlett 2000).

A number of transcriptional factors (TFs) are suggested to play a role in maintaining the multipotent network of NSPCs based on loss of function or gain of function studies (Ahmed et al 2009, Favaro et al 2009, Kim et al 2007b, Lefebvre et al 2007, Lugert et al 2010, Marei & Ahmed 2013), although the role of transcriptional cascades in neuron production in the adult neurogenic regions is relatively unknown compared to that occurring in embryonic neurogenesis (Hsieh 2012). However, a series of TFs are known to be expressed in neurogenic niches. TFs include Sox2, which is found to be essential for NSPC proliferation and maintenance (Favaro et al 2009). Ascl1, a basic helix–loop–helix (bHLH) transcription factor that has been implicated in the induction of neuronal fate of NSPCs but not in the stimulation of glial differentiation (Kim et al 2011, Kim et al 2007b), and Hes5, a bHLH transcription factor that has been proposed to be a direct target of Notch signalling in regulation of cell growth (Ables et al 2011, Lathia et al 2008, Lugert et al 2010).

1.7.2.4.2.1 Cystatin C

As an endogenous cysteine protease inhibitor, cystatin C (CysC) (Bobek & Levine 1992) is ubiquitously produced and secreted by various tissues. It is found in most mammalian body fluids, and at particularly high concentrations in the CSF (Abrahamson et al 1986, Turk et al 2008). In the CNS, CysC is abundantly expressed (Hakansson et al 1996), and is produced by neurons, astrocytes and microglial cells (Miyake et al 1996, Palm et al 1995, Yasuhara et al 1993). CysC is a hydrophilic protein containing 120 amino - acid residues with two intramolecular disulfide bridges that are mainly involved in modulating the activity of cysteine proteases (Otto & Schirmeister 1997). CysC inhibits lysosomal cathepsins especially cathepsin B, H,

K, L (Abrahamson 1994), caspases (Thornberry 1997) as well as cytosolic calpains (Turk et al 1997). Cysteine proteases play important roles in various biological processes including protein degradation, cell proliferation, differentiation and apoptosis (Berdowska & Siewinski 2000, Chapman et al 1997, Grzelakowska-Sztabert 1998). Thus precise control of cysteine protease activities by inhibitors such as CysC is essential for normal cell function. CysC, like other cystatins, is an exosite binding inhibitor that binds close to the active site of the cysteine protease, but does not interact with the active site directly (Bode & Huber 2000). CysC is suggested to participate in a wild range of biological process, such as the regulation of inflammatory responses (Kopitar-Jerala 2006, Magister & Kos 2013, Warfel et al 1987), cell proliferation and growth (Sun 1989, Tavera et al 1992), and neural repair (Suzuki et al 2014a, Watanabe et al 2014, Zhong et al 2013).

CysC null mice exhibit decreased basal levels of neurogenesis and impaired proliferation and migration of newly generated granule cells in the dentate gyrus following seizures (Pirttila et al 2005, Taupin et al 2000). This suggests that there is a possible relationship between CysC and adult neurogenesis (Kaur & Levy 2012). Dividing NSPCs in vitro and in vivo were both found to be immunoreactive for CysC antibody, while combined delivery of CysC and FGF-2 stimulated increased neurogenesis in the dentate gyrus (Taupin et al 2000). These results raise a possibility the CysC may act as autocrine factor for NSPC growth (Taupin et al 2000). Proteome analysis indicates that CysC is secreted into the conditioned medium by cells during NSPC proliferation (Dahl et al 2003, Taupin et al 2000). CysC purified from NSPC conditioned medium was shown to promote NSPC proliferation in vitro (Dahl et al 2004, Taupin et al 2000) although CysC released by ESCs into the conditioned

medium does not display a stimulatory effect on NSPC proliferation. Nevertheless, the concentration of exogenous CysC that was used in these experiments was 20 ng/ml which may not have been adequate to increase NSPC proliferation (Tham et al 2010). Moreover, CysC purified from adult NSPC was reported to induce ES cells to differentiate into NSPCs which retain the ability to self-renew and exhibit multi-potency in vitro (Kato et al 2006). Furthermore, a higher proportion of β III tubulin (neuron marker), GFAP (astrocyte marker) and MBP (oligodendrocyte marker) positive cells was observed in ES-NSPC cultures treated with purified CysC compared to a control group (Kato et al 2006). Besides, CysC was shown to activate GFAP promoter and to be expressed in astrocyte progenitors during development (Kumada et al 2004). Therefore, CysC may contribute to both embryonic and adult NSPC fate and behaviour.

Up-regulation of CysC protein and mRNA levels in neurogenic regions of the dentate gyrus of the hippocampus were reported following brain injury and seizure (Aronica et al 2001, Hendriksen et al 2001, Lukasiuk et al 2002). Interestingly, increased CysC expression occurs at the time when neurogenesis is maximal (Kumada et al 2004, Nairismagi et al 2004, Parent et al 1997). As CysC is involved in neurogenesis during development, it may also be involved in neurogenesis after injury. Indeed, proliferation of adult NSPCs in SGZ was decreased in CysC knockout mice compared to litter-mate controls after brain injury (Pirttila et al 2005).

However, the mechanism underlying CysC - induced NSPC proliferation as well as ESC – NSPC differentiation is still uncertain (de Azevedo-Pereira et al 2011) . CysC reportedly may regulate cell proliferation independent of its effect on cysteine

proteases (Sun 1989, Tavera et al 1992), possibly by acting as a growth factor to stimulate NSPC proliferation (Taupin et al 2000). However, cysteine proteases are also supposed to be an effector of cell proliferation and differentiation (Egberts et al 2004, Joyce et al 2004, Watkinson 1999, Yadaiah et al 2013b), which raises the possibility that regulation of cysteine protease activities by cysteine protease inhibitors may also affect NSPC proliferation and differentiation. Mouse ESCs have been shown to differentiate into more NSPCs by addition of cysteine protease inhibitors such as CysC and E-64 (de Azevedo-Pereira et al 2011), and higher β III tubulin – positive cells (neurons or neuronal progenitors) were found in ESC cultures which were treated with E-64 (de Azevedo-Pereira et al 2011). However, the mechanism of induced differentiation of mouse ESC into NSPC and neurons by inhibition of cysteine proteases may be distinct from CysC - induced neural differentiation (de Azevedo-Pereira et al 2011). Interestingly, CysC increases the number of NSPCs as well as GFAP-positive and nestin-positive cells from brain ESC culture. Nevertheless, the increased number of GFAP cells reportedly does not seem to be due to inhibition of cysteine proteases by CysC (Hasegawa et al 2007). Therefore, the basis of CysC induced proliferation and differentiation of NSPC needs further investigation.

1.7.2.4.2.2 Neurogenin 2

Neurogenin 2 (Ngn2) is a proneural factor that was originally found to improve neuronal differentiation in brain and spinal cord (Li et al 2012b, Ma et al 1996, Sommer et al 1996). Ngn2 is expression in both CNS and PNS during embryonic neural development (Bertrand et al 2002, Chapouton et al 2001, Parras et al 2002, Ross et al 2003, Sommer et al 1996). Ngn2 induces neuronal determination of NSPC

(Scardigli et al 2001, Simmons et al 2001, Thoma et al 2012), and has been suggested to participate in neuronal-type and morphological specification (Florio et al 2012, Hand et al 2005, Hand & Polleux 2011, Kele et al 2006, Ma et al 2008c, Park et al 2008a) as well as in the control neuron migration (Heng et al 2008). However, Ngn2 suppresses progenitor cell cycle progression during neural development and maturation while promoting neuronal differentiation (Florio et al 2012).

Ngn2 null mutants survive a few days postnatally (Andersson et al 2006), although the vast majority of Ngn2 null animals die around birth (Fode et al 1998, Fode et al 2000, Hand et al 2005). Nonetheless, in the absence of Ngn2, animals exhibit abnormalities including decreased cerebellum volume, a lobulation defect in the cerebral hemisperes and reduced numbers of NSPCs (Florio et al 2012). This suggests a crucial role of Ngn2 in embryonic neurogenesis. Ngn2 activity declines at a very late stage of embryonic development (Li et al 2012b). Nevertheless Ngn2 is also expressed by NSPCs in adult neurogenic niches (Ozen et al 2007, Roybon et al 2009b). This suggests that Ngn2 also plays an important role in adult neurogenesis (Galichet et al 2008, Ozen et al 2007, Roybon et al 2009b). Interestingly, Ngn2 also promotes the genesis of neurons from non-neural cell types such as fibroblasts (Liu et al 2013, Yan et al 2001a). Moreover, NSPCs transduced with Ngn2 displayed improved survival and differentiation after transplantation (Yi et al 2008) and endogenous NSPCs transfected with Ngn2 carrying retroviruses showed neuroblast-like morphology (Rogelius et al 2008). Therefore, investigation into function of Ngn2 in NSPC proliferation and differentiation may be helpful for understanding the mechanism of induction of neurons for stem cell therapies.

1.7.2.4.3 An in - vitro model for studying NSPCs: neurospheres

Embryonic stem cells (ESCs) (Reynolds & Weiss 1992) and neural stem cells as well as their progeny (NSPCs), may be derived from the CNS of animals of any age and induced to proliferate in response to growth factors (Reynolds & Weiss 1992, Vescovi et al 1993) or to differentiate when they are exposed to specific culture conditions (Helgason & Miller 2013, Reynolds & Weiss 1992) in vitro. NSPCs have the capacity for self-renewal through their selective response to EGF as well as to FGF in vitro. NSPC cultures can be established from the V-SVZ and SGZ-DG or other regions of the fetus or adult brain (Bordiuk et al 2014, Liu & Martin 2003, Lois & Alvarez-Buylla 1993, Palmer et al 1997, Reynolds & Weiss 1992, Richards et al 1992, Weiss et al 1996). The culture medium is usually serum free, but supplemented with B27 (containing retinoic acid and insulin) or N2 (containing insulin and transferrin) in order to select for expanding NSPCs (Gritti et al 1996, Reynolds & Weiss 1996, Vescovi et al 1993). NSPCs grow in vitro as spherical floating cluster known as neurospheres, but they can also be cultured as adherent cells (Palmer et al 1997, Potten & Loeffler 1990, Ray et al 1993, Weiss et al 1996). Upon removal of the growth factors, the neurospheres can generate three major types of CNS cells (Reynolds & Weiss 1992). Neurospheres can also be derived from ESCs (Louis & Reynolds 2005, Tropepe et al 2001, Ying et al 2002). A single neurosphere is generally thought to clonally expand from a single stem cell. Nonetheless the evidence indicates that both stem and progenitor cells can proliferate to form neurospheres (Reynolds & Rietze 2005).

The neurosphere culture system was the primarily in vitro NSPC culture system to illustrate the existence of a group of NSPCs in the adult brain with the properties of

self-renew and multipotency (Reynolds & Weiss 1992, Reynolds & Weiss 1996). The neurosphere culture model is a useful technique that allowed evaluation of NSPC proliferation, self-renewal and multipotency via testing on neurosphere forming or differentiation potential (Gritti et al 1996, Rietze et al 2001, Tropepe et al 1999, Uchida et al 2000). In addition, the neurosphere culture system allows the study of neural development and adult neurogenesis in vitro (Alexson et al 2006, Jensen & Parmar 2006), and especially to investigate intrinsic characteristics of NSPCs at different developmental stages (Falk et al 2002, Jensen & Parmar 2006, Klein et al 2005). In addition, this in vitro NSPC culture model has been used to study the differentiation potential of NSPCs upon exposure to specific extrinsic factors (Ben-Hur et al 2003, Jensen & Parmar 2006). Importantly, neurospheres in biomedical research can be applied to drug test screening, or to identify factors affecting NSPC fate. This is due to the possibility of culturing a pool of specialized neural cells (Galli 2013, Singec et al 2006), and the ability to efficiently manipulate intrinsic properties of NSPC by transduction (Falk et al 2002, Hack et al 2004, Heins et al 2002). Furthermore, neurosphere-derived cells have potential applications in stem cell therapy for neurological disorders such as neurodegenerative diseases because they may provide a sustainable cell source for neural regeneration (Gil-Perotin et al 2013).

1.8 Neurogenesis in neurodegenerative disease

A number of studies have reported that adult neurogenesis is affected by pathological situations including stroke (Greenberg 2007), seizure (Parent et al 1997), acute trauma (Gao et al 2009, Miles & Kernie 2008) and neurodegenerative disease (Jin et al 2004c, Winner et al 2011). Neurodegenerative diseases are a broad range of disorders that have the common features including the progressive structural and functional loss of

neurons and glial cells in the brain and spinal cord (Winner et al 2011). Chronic neurodegeneration may exert various effects on NSPC maintenance (Kandasamy et al 2010), proliferation (Hoglinger et al 2004, Jin et al 2004c), survival and functional integration (Winner et al 2011). Furthermore, altered or even impaired adult neurogenesis has been described in models of neurodegenerative diseases such as Alzheimer's disease (AD) (Jin et al 2004c, Mirochnic et al 2009, Rodriguez et al 2008), Parkinson's disease (PD) (Crews et al 2008, Nuber et al 2008, Winner et al 2004) and Huntington's disease (HD) (Kandasamy et al 2010, Kohl et al 2007). However, whether deficits in neurogenesis contribute to neurodegenerative diseases still remains unknown, although impaired olfaction and hippocampus associated cognitive and emotional deficits are commonly identified in variety of many neurodegenerative diseases (Winner et al 2011).

1.8.1 Neurogenesis in Alzheimer's disease

Adult neurogenesis was suggested to contribute to learning and memory (Deng et al 2010). The rate of neurogenesis decreases with age, and seems to be further affected by AD pathogenesis (Jin et al 2004c, Kuhn et al 1996). A number of studies have used AD transgenic model animals to investigate this effect (Jin et al 2004a, Perry et al 2012, Yu et al 2009), although the mechanism underlying the neurogenesis response to AD pathology is still unknown (Jin et al 2004a, Martinez-Canabal 2014, Perry et al 2012, Yu et al 2009). Altered adult neurogenesis has been reported in AD transgenic animal models in the neurogenic niches such as the V-SVZ and DG-SGZ depending on post-mortem analysis (Marlatt & Lucassen 2010). Post-mortem studies of neurogenesis found enhanced expression of immature neuronal markers in the brain of AD patients (Jin et al 2004c). Furthermore, increased neurogenesis has been

identified in several AD transgenic mouse models (Jin et al 2004a, Lopez-Toledano & Shelanski 2007, Yu et al 2009). Nonetheless, some other studies challenge these observations and suggest that there are no changes or even decreased NSPC proliferation, in patients with AD (Boekhoorn et al 2006, Donovan et al 2006, Moon et al 2014, Rodriguez et al 2008).

However, the conflicting results may be due to combination of factors. Adult neurogenesis may change in response to AD pathology (Perry et al 2012, Yu et al 2009) such as Aβ deposition and synaptic loss (Kanemoto et al 2014, Perry et al 2012). Therefore, the increased NSPC proliferation found in AD cases may be a compensatory response to the pathologic changes of AD. Different types of AD pathology such as cholinergic degeneration may have detrimental effects on adult neurogenesis (Perry et al 2012). In addition, NSPC proliferation was reported to increase prior to the appearance of Aβ pathology, although the viability of NSPCs declines over the time course of AD pathogenesis (Chishti et al 2001, Kanemoto et al 2014). Studies of neurogenesis in AD are dependent on the use of AD transgenic mouse models involving knock-in of various FAD-related human APP mutations, often in combination with PS (PS1 or PS2) or tau mutations (Chishti et al 2001, Citron et al 1998, Mullan et al 1992, Oddo et al 2003). Indeed, these APP variants, or different PS species or other critical molecules may account, at least to some degree, for the observed divergence in neurogenic fate in the different AD transgenic models from these studies (Lazarov & Marr 2010). In addition, different APP or PS mutations or other gene insertions may exert different effects on adult neurogenesis, which also may be a cause of the inconsistent results obtained among these AD animal studies.

1.8.1.1 Role of PS on adult neurogenesis

PS is the catalytic domain of the γ - secretase complex (De Strooper et al 1998), which is needed for proteolysis of a number of transmembrane proteins including Notch and β - catenin as well as APP (De Strooper 2003). PS mutants that are especially common in PS1 contribute to Aβ pathology by increasing the production of Aβ cleaved at position 42 (De Strooper et al 1998). PSs play a role in neural development or adult neurogenesis (Chen et al 2008, Shen et al 1997, Wong et al 1997). However, the precise mechanism by which PSs influence development is still unknown (Lazarov & Marr 2010). However, PS1 null mice display severe abnormalities in somitogenesis as well as neurogenesis in the brain (Shen et al 1997, Wong et al 1997). PS1 null mice do not survive postnatally, which has complicated studies of PS1 function in postnatal development (Shen et al 1997, Wong et al 1997). For this reason, investigations into the role of PS in neurogenesis in vivo have been carried out in PS1 transgenic mice (Lazarov & Marr 2010) or mice with a conditional deletion of the PS1 gene (Chen et al 2008, Feng et al 2001). In contrast, PS2 null mice show no obvious deficit, although inactivation of both PS2 and PS1 is embryonic lethal, suggesting functional redundancy between PS1 and PS2 (Donoviel et al 1999).

PS1 is expressed in NSPCs from neurogenic regions in the adult brain (Wen et al 2002a) and wild-type PS1 overexpression has been found to increase neurogenesis (Wen et al 2002b). Mice carrying a partial deletion of PS1 show no obvious difference in NSPC proliferation, which may possibly be explained by the compensatory effect exerted by PS2 (Chen et al 2008, Donoviel et al 1999). Nevertheless, mice harbouring a conditional PS1 deletion, but a conventional PS2 deletion, exhibit both enhanced NSPC proliferation and neuronal differentiation

(Chen et al 2008). This increased NSPC proliferation and differentiation may be due to the pathological stimulation, such as cortical neuron loss in mice with partial deletion of PS1 and completely null of PS2 (Chen et al 2008).

Surprisingly, mice with a single mutation in PS1 have only a minor reduction in the number of neuroblasts. Nevertheless, a dramatic decline in neuroblast number was observed in APP/PS1 double knock-in mice bearing a FAD mutation (Zhang et al 2007a). Most murine studies link FAD-associated PS1 mutations with impaired neurogenesis. For example, a more recent study reported that endogenous expression of a FAD-linked PS1 variants may be sufficient to impair NSPC proliferation, differentiation and survival (Veeraraghavalu et al 2010, Veeraraghavalu et al 2013), and another study suggested that expression of several PS1 mutants in NSPCs derived from the V-SVZ leads to a deficit in self-renewal and premature differentiation towards a neuronal fate in vitro (Gadadhar et al 2011, Lee et al 1997, Thinakaran et al 1996). In addition, PS may act as regulators of adult neurogenesis possibly via Notch signaling (De Strooper et al 1999, Wong et al 1997), therefore the FAD-related PS1 variants may cause a loss of PS1 function in neurogenesis regulation (De Strooper 2007).

1.8.1.2 Role of tau in neurogenesis

The pathological tau isoforms found in AD also have been suggested to have an impact on neurogenesis (Pristera et al 2013) although comparatively fewer studies have focused on that idea. A transgenic mouse model conditionally expressing a fragment of hyper - phosphorylated tau had reduced NSPC proliferation and survival and increased cell death in the dentate gyrus of hippocampus (Pristera et al 2013).

However, another study using animals containing a human tau isoform exhibited increased neurogenesis and cell cycle events (Schindowski et al 2008). This opposite result may be due to different pathological forms of tau being employed in the studies, therefore the impact of tau in neurogenesis warrants further study.

1.9 Stem cell therapy for neurodegenerative disease

Neuron and glial cell loss occurs in many neurodegenerative diseases (Lindvall & Kokaia 2010, Lunn et al 2011). Acute neurodegenerative disorders, caused for example by insults such as stroke or tramautic brain injury, result in a loss of neurons at the specific injury sites (Lindvall & Kokaia 2010). Chronic neurodegeneration involving a slower progressive time course is often characterized by gradual loss of a particular subtypes of neurons (such as dopamine neurons in PD) or by a continuous degeneration of a wide range of neuronal subtypes as occurs in AD (Lindvall & Kokaia 2010).

Stem cell - based therapies have been proposed for the treatment of neurodegenerative diseases as a means of replacing impaired or dysfunctional neurons. Stem cells exhibit either pluripotency or multipotency, and have the capacity to self-renew and proliferate (Reynolds & Weiss 1992, Thomson et al 1998). Stem cell based – approaches are dependent on two fundamental concepts: 1) manipulation of endogenous neurogenesis to generate new neurons at the neurogenic niches. Neurons can migrate toward the injured sites to improve self-repair; and 2) transplantation of stem cells into the sites of damage either to replace loss or defective neurons or other types of cells, or to deliver growth factors to the damage site to promote regenerations (Lindvall et al 2012).

Stem and progenitor cells derived from different sources can be used to treat neurodegenerative disorders. They may be stem cells or neural stem cells or progenitor cells (Lunn et al 2011). Stem cells are a group of cells which have the ability to undergo self -renewal consistently and possess pluripotent behaviour and are able to differentiate into a broad range of cell types which give rise to cells found in various tissues and organs (Kim & de Vellis 2009). Two types of mammalian pluripotent stem cells have been identified, embryonic stem cells (ESCs) isolated from the inner cell mass of blastocysts (Thomson et al 1998) and embryonic germ cells (EGCs) obtained from post-implantation embryos (Donovan 2001, Shamblott et al 1998). However, multipotent NSPCs can be derived from fetal or adult neural tissues, or differentiate from ESCs or EGCs (Gaspard & Vanderhaeghen 2010, Wichterle et al 2002). Alternatively, mesenchymal stem cells (MSCs) that are isolated from adult bone marrow have been though of as a source to produce the cells with multipotency and ability to self-renew (Satija et al 2009). MSCs differentiate into osteoblasts, chondrocytes as well as adipocytes in natural conditions. They are also able to trans-differentiate into neural specific lineages (Satija et al 2009). MSCs are an autologous cell source that may tolerate immune rejection after transplantation, but MSCs derived from patients with a genetic disease might not be expected to be a source of functional cells for transplantation (Cho et al 2010a, Choi et al 2010).

Recently, a new class of pluripotent stem cell has been developed by conerting adult somatic cells such as skin fibroblasts or peripheral blood into ESC-like cells via the introduction of embryogenesis-related genes (Gianotti-Sommer et al 2008, Park et al 2008c, Takahashi et al 2007, Yu et al 2007). These cells are known as induced pluripotent stem cells (iPSCs). iPSCs are created by reprogramming somatic cells to

convert them into ESC-like cells through the introduction of selected transcription factors, such as Oct 3/4, Klf, Sox2 and c-Myc (Yamanaka 2008). Various combinations of factors are used to reprogram of fibroblasts. The factors are delivered through a vector, virus, protein or RNA-based approaches (Cho et al 2010b, Hanley et al 2010, Judson et al 2009, Yakubov et al 2010). iPSC technology allows cells to be induced to an ESC - like state to generate any cell-type, such as major cell types, that are then potentially useful for clinically therapeutic applications (Park & Eve 2009, Srivastava et al 2008, Yamanaka 2008). In addition, this technology provides an endless supply of stem cells from differentiated somatic cells of patients who are affected by neurological diseases (Kiskinis & Eggan 2010, Park et al 2008b, Salewski et al 2010). iPSCs are therefore patient-specific and thereby minimize the clearance of host rejection (Araki et al 2013) compare to traditional ESCs which are derived from other source of donors. Importantly, the programmed differentiation of patient iPSCs can possibly be developed to model the pathogenesis of human disease for understanding the underlying mechanism as well as for development of potential therapeutic strategies (Lunn et al 2011). However, there are still major obstacles to overcome before iPSCs become clinically acceptable for the treatment of human neural disorders. Firstly, cell reprogramming by either transcription factors or expressed proteins may introduce immunogenicity (Araki et al 2013, Zhao et al 2011) and tumorigenicity (Duinsbergen et al 2009, Lee et al 2013a, Miura et al 2009, Nakagawa et al 2008, Yu et al 2013) in recipients. Secondly, and importantly, the efficiency of generating specific neural cell type for the treatment of disease, and integration of iPSCs- derived neural cells into existing neural circuits, should be taken into consideration (Cai et al 2010, Tsuji et al 2010, Yu et al 2013).

In summary, stem cell based therapies are potentially a promising treatment for neurological disorders, as various preclinical studies have illustrated functional recovery in animal models after transplantation of neurons derived form ESCs or iPSCs (Hargus et al 2010, Ma et al 2012b, Roy et al 2006, Wernig et al 2008). However, the basis of improvement, whether it is due to the repair and reorganization of the damaged neural network and the neurotrophic supports, or through other mechanisms, requires further clarification. At least four issues have to be considered regarding safety and efficacy when stem cell therapy is applied to humans: 1) long-term survival and phenotypic and functional integration of the graft (either neurons or glial cells); 2) high purity of the graft to avoid unexpected interaction with circuitry; 3) tumours which may arise from the grafts; 4) and compatibility with the native immune system (Kim & de Vellis 2009).

1.10 APP and neurogenesis

Several studies showed that APP expression is increased during period of neuronal differentiation in embryonic CNS development (Clarris et al 1995, Hung et al 1992, Salbaum & Ruddle 1994). In addition, abundant APP expression in radial glial cells of developing fetus mouse brain can be detected by immunocytochemistry (Trapp & Hauer 1994). Thus APP has been suggested to play a role in embryonic CNS development (Clarris et al 1997, Hung et al 1992). There are many studies on the role of APP or its homologues, APLP1 and APLP2 in CNS development and neurogenesis (Bergmans et al 2010, Chen & Tang 2006, Heber et al 2000, Kanemoto et al 2014, Ma et al 2008b, Shariati et al 2013, Young-Pearse et al 2007). However, the present roles of these proteins remain unclear.

APP null mice do not exhibit extremely severe brain deficiencies, although they have reduced brain weight and other neuronal function impairments such as hypersensitivity to seizure-inducing stimuli (Dawson et al 1999, Muller et al 1994, Steinbach et al 1998, Zheng et al 1996, Zheng et al 1995). Interestingly, NSPCs isolated from APP KO mice at embryonic 14 days showed increased cell proliferation (Zheng et al 1996, Zheng et al 1995). However, another study indicates an opposite result, with decreased proliferation of NSPCs obtained from postnatal mice lacking APP, compare to the corresponding wild-type controls (Hu et al 2013). The differences may be due to the investigations being carried out on NSPCs cultured from different developmental time points, i.e. one from an embryonic stage, and the other from a postnatal stage (Hu et al 2013, Zheng et al 1996, Zheng et al 1995). The reason for the discrepancy in the results is unknown. However, the in vitro neurosphere culture system is very sensitive to culture methods and environment, which may change the sensitivity or likelihood of NSPC proliferation and differentiation (Jensen & Parmar 2006). In addition, a recent in vivo study reported increased NSPC proliferation in the adult hippocampus of APP null mice compared to the WT control mice (Wang et al 2014). A physiological deficiency in APP null mice (Dawson et al 1999, Steinbach et al 1998, Zheng et al 1996, Zheng et al 1995) may have been the cause of the increased neurogenesis (Hayon et al 2013, Jin et al 2004b, Jin et al 2004c, Parent et al 1997). For example, mice lacking APP are hypersensitive to seizures (Steinbach et al 1998), and this increased seizure activity may induce enhanced neurogenesis on cell proliferation at neurogenic niches (Parent et al 1997, Shetty et al 2012). Thus, the definitive role of APP in NSPC proliferation remains unclear.

One study has found a reduced number of spines on dendrites of cortical and hippocampal neurons in APP-KO mice (Lee et al 2010). In addition, APLP2 is reported to control differentiation of neural stem cells during cortical development (Shariati et al 2013). Furthermore, early postnatal death and serious neuroanatomical abnormalities are observed in APP/APLP2 and APLP1/APLP2 double knockout mice (Chen & Tang 2006, Heber et al 2000, von Koch et al 1997). This suggests that APP and its homologue probably play a critical role in embryonic and adult neurogenesis, and it also suggests a possible compensatory relationship between APP and APLP2 (Heber et al 2000, von Koch et al 1997). APP/APLP1/APLP2 triple knockout (APP tKO) mice have been created for deeper investigations of the function of the APP family in CNS development. Unfortunately, APP tKO mice are perinatally lethal (Herms et al 2004). However, NSPCs derived from APP tKO ESCs display normal neuronal differentiation and generation of active excitatory synapses in vitro, and the APP tKO neurons are morphologically intact and exhibit normal migration in vivo (Bergmans et al 2010). The reason for the death of the embryo around birth is unknown (Bergmans et al 2010). As the NSPC culture system is sensitive to culture conditions, the properties of cells to differentiate may change in response to the method and the environmental conditions applied in culture (Jensen & Parmar 2006). The reason why NSPCs response differently in vitro to in vivo may be that cells in vivo are regulated by a more complicated system of cellular interaction than cells in vitro (Jensen & Parmar 2006). To identify the role of APP in NSPC differentiation, more studies are required.

Numerous reports suggest that APP metabolites may be involved in the regulation of adult neurogenesis, especially in the modulation of NSPC proliferation or

differentiation. For example, a soluble form of APP, sAPPα has been reported to have a trophic effect on NSPC behaviour (Baratchi et al 2012, Caille et al 2004, Demars et al 2011, Ohsawa et al 1999). However, little is known about how sAPPα achieves the observed effects on NSPC division.

Aβ is another proposed regulator of NSPC proliferation and differentiation. However, the studies reporting an effect of Aβ are controversial or even conflicting (Chen & Dong 2009, Haughey et al 2002, Kanemoto et al 2014, Lee et al 2013b, Sotthibundhu et al 2009, Zheng et al 2013). These contradictions may be due to the different forms of Aβ used in the studies. Furthermore, various ways that Aβ is reconstituted in each study may alter the Aβ configuration, and subsequently affect NSPC behaviour. Notably, synthetic Aβ reportedly exerts different effects on NSPC biology than the endogenous Aβ (Nicolas & Hassan 2014).

The role of the intracellular domain of APP (AICD) in NSPC regulation remains unclear as well. By analogy with the NICD, AICD is suggested to act as a transcriptional factor that negatively regulates the gene encoding the EGFR, which is known to stimulate NSPC proliferation (Ayuso-Sacido et al 2010, Zhang et al 2007b). Besides, a study on APP KO mice that express AICD exhibit reduced proliferation as well as reduced survival of NSPC in the DG-SGZ (Ghosal et al 2010). In addition, one study suggests that transient axonal glycoprotein 1, a neural cell adhesion protein, interacts with APP to initiate AICD release to negatively modulate neurogenesis (Ma et al 2008b). However, another study does not agree with the conclusion that AICD participates in the nuclear signaling pathway (Hebert et al 2006), highlighting further studies in this area.

1.11 Hypothesis and aims of the study

APP has been well studied for its role in AD pathogenesis. Nevertheless, the functions of APP still remain a mystery, despite intensive research in this area. Understanding the biological function of APP may contribute to our understanding of the role of APP in AD and the normal biology of brain.

APP is likely to participate in a variety of neuronal and non-neuronal processes including synaptogenesis, neurite outgrowth and the regulation of a number of genes vital for cell viability (Allinquant et al 1995, Bush et al 1990, Lee et al 2009, Murayama et al 1996). APP expression occurs coordinately with stem cell maintenance in the developing rat olfactory system, and in neural stem cells (radial glial) during embryonic development (Clarris et al 1995, Trapp & Hauer 1994). Furthermore, APP is expressed in both neuroblasts as well as in neurons when NSPCs proliferate and differentiate (Clarris et al 1995, Fukuchi et al 1992, Masliah et al 1992). As adult neurogenesis is suggested to play a role in normal brain function (Akers et al 2014, Dupret et al 2007, Snyder et al 2011), APP could potentially regulate neural stem cell development. AD treatments that alter APP processing could potentially affect normal brain function. For this reason, it is vital to understand whether APP or APP metabolites exert effects on adult neurogenesis.

1.11.1 Hypothesis

The central hypothesis presented in this study is that APP regulates NSPC proliferation and differentiation. It is further hypothesized that APP – induced NSPC proliferation is due to the production of a secreted growth factor.

1.11.2 Aims

To test this hypothesis, the following specific aims of this study have been covered in each chapter:

1. To examine the effect of APP on NSPC proliferation. The proliferation of NSPCs obtained from APP overexpressing mice model (Tg2576 mice) and APP null mice (APP KO) grown in culture was compared with that of the corresponding background strain control.

2. To identify mechanisms by which APP may affect NSPC proliferation. Conditioned medium collected from Tg2576 NSPC culture and APP KO culture was analyzed to determine whether APP expression drives production of any secreted factors that could promote NSPC proliferation.

3. To examine the effect of APP on NSPC differentiation. NSPCs were derived from APP overexpressing Tg2576 mice and APP null mice and from background strain control mice. Upon differentiation of NSPC, the production of neurons, astrocytes as well as oligodendrocyte progenitors was examined.

4. To identify mechanisms by which APP may affect NSPC differentiation. The NSPC cultures were treated with the secreted fragments of APP (Aβ and sAPPα). The expression of neuronal, astrocytic and oligodendrocyte progenitor markers in the cultures was examined.

Chapter 2

Materials and Methods

2.1 Materials

All materials and suppliers of materials used in this study are shown in Table 2.1.

2.1.1 Buffers, solutions and cell culture media

All solutions and buffers used in this study are listed in Table 2.2.

2.1.2 Antibodies used in this study

All the primary antibodies and all the secondary antibodies, and supplies of antibodies are shown in Table 2.3.

2.2 Methods

2.2.1 Neural stem/progenitor cell (NSPC) culture

NSPC cultures were prepared from the cerebral cortices of new born (postnatal day 0, P_0) human APP overexpressing mice (APPSW Tg2576, Taconic Farms Inc., Hudson, NY) and from the corresponding wild type littermate controls (C57Bl6 × SJL), as well as from APP knockout (APP KO, Jackson laboratory, Bar Harbor, ME) mice and the corresponding background wild type controls (C57Bl6). Brain cortices were dissected away from the cerebellum, and then the meninges and hippocampus also removed. Cortical tissue was then incubated in 1×TrypLE Express for 10 minutes at 37°C. The cortices were mechanically dissociated with 1000 μL fine tips, and then passed through a 40 μm cell strainer (BD Bioscience, North Ryde, Australia) to remove non-separated cells. NSPCs were plated at a density of 20,000 cells/mL in T75 cell culture flasks containing proliferation medium (Table 2.2). NSPCs were grown as neurospheres in suspension and incubated in a humidified incubator at 37°C with 5% CO_2. After culturing for 7 days, the neurospheres were collected by

centrifugation at 500 × g for 5 min at room temperature. Conditioned medium was collected and stored at -80°C for further analysis. Neurospheres pellets were passaged by mechanical dissociation with 200 µL fine tips. Cells were stained with Trypan Blue and counted under a Bright-Line^{TM} haemocytometer (Sigma-Aldrich Pty. Ltd. Castle Hill, Australia). The dissociated cells were passaged and either reseeded as suspension cultures or replated as adherent cultures for further analysis. All cultures were incubated in a humidified incubator at 37 °C with 5% CO_2.

For proliferation and differentiation assays, the NSPCs were cultured adherently on poly-L-lysine coated plates in either proliferation or differentiation medium (Table 2.2).

2.2.2 AlamarBlue Assay

The cell viability (expressed as an index of the number of living cells) was measured by an alamarBlue assay. The isolated cells were plated adherently in 100 µL of fresh proliferation medium on poly-L-lysine coated 96-well-plates at a density of 2000 cells/well, and to each well was added 100 µL of medium containing the treatment. The cells were then incubated for various time periods, after which 20 µl of alamarBlue reagent (Hayashi et al 1994) was added into each well and the plates incubated for another 4 hours. The fluorescence intensity was measured using a FLUOstar Optima microplate fluorescence plate reader (BMG Labtech GmbH Ortenberg, Germany) at an excitation wavelength of 540 nm and an emission wavelength of 590 nm. Cell number was presumed to be proportional to the relative fluorescence intensity.

2.2.3 EdU incorporation assay of cell proliferation

Cells were cultured adherently on a coverslip in proliferation media at a density of 20,000 cells per well in a 24 well-plate in which each well contained one coverslip. Plates were followed by incubated for up to 4 days, after which cells were incubated with 10μM EdU for 8 hours before fixing in 4% (w/v) paraformaldehyde in PBS for 15 min. Cells were permeabilized with 0.03% (v/v) Triton X-100 in PBS for 5 min, and then and then blocked in 4% goat serum in PBS for 20 min. The fixed cells were immunostained with polyclonal anti-Nestin antibody 1:5000 diluted in 2% goat serum in PBS and then incubated with a goat anti-mouse IgG conjugated to Alexa Fluor 488 (1:1000 diluted in 2% goat serum in PBS). EdU was visualized using the AlexaFluor-594 Click-iT® EdU Cell Proliferation Assay Kit immediately after immunocytochemistry. Cells on the coverslip were incubated at 20°C-23°C for 15 min in PBS/0.5% (v/v) Triton X-100 and then transferred to the EdU developing cocktail (10% v/v Click-iT® reaction buffer, 10% v/v Click-iT® reaction buffer additive, 80% v/v $CuSO_4$), incubated in the dark at 20°C–23°C for 30 min, and washed in PBS. Cell nuclei were counterstained by poststaining with 4′,6-diamidino-2-phenylindole (DAPI) at 1:7000 dilution before coverslips were mounted in fluorescence mounting medium (DAKO, Cytomation). An estimate of cell proliferation was given by the proportion of cells positive for EdU staining in the population of cells positive for both of DAPI and Nestin (Alexa Fluor 488).

2.2.4 NSPC differentiation and cell fixation

Neurospheres were mechanically dissociated, and then isolated cells were plated at a density of 10^5 cells/well on poly-L-lysine coated coverslips in differentiation medium

(Table 2.2) in 24-well plates. After 5-14 days in culture, the cells were washed once with pre-warmed (37°C) phosphate-buffered saline (PBS) and then by fixed with 4% (w/v) paraformaldehyde in PBS for 10 minutes. Fixed cells were washed with PBS three times and kept in PBS containing 0.02% (w/v) sodium azide at 4°C for immunocytochemistry (Chapter 4).

2.2.5 Immunoblotting

Samples had a one fifth volume of 5 × Laemmli sample buffer (Table 2.2) added and heated to 95°C for 5 minutes. Mixtures were centrifuged at 10,000 rpm (microcentrifuge) for 5 seconds. Samples were loaded onto 8 or 12% sodium dodecyl sulfate Tris-glycine polyacrylamide (SDS-PAGE) gels to separate protein according to their size by electrophoresis. Proteins were then transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes. The PVDF membrane was blocked in 5% (w/v) skim milk powder in 50 mM Tris-buffered saline (pH 8) containing 0.05% (v/v) Tween 20 (TBS-T) for one hour. Specific proteins were detected by incubation with primary antibody at the appropriate dilution in 5% (w/v) skim milk TBS-T buffer at 4°C overnight. Membranes were washed for three or four times in TBS-T before incubation with HRP-conjugated secondary antibody of the appropriate dilution in 5% (w/v) skim milk TBS-T at room temperature for one hour. Chemiluminescence was measured using a CHEMI-SMART 5000 image acquisition system and images were captured using Chemi-Capt version 5001 (Vilber-Lourmat, Eberhardzell, Germany). For quantification of immunoreactivity, the integrated density of luminescence for each band was analyzed using ImageJ version 1.46.

Table 2.1 Materials and supplies of materials used in this study

Material	**Supplier**
30% Acrylamide/Bis solution	Bio Rad Laboratories Pty. Ltd., Gladesville, Australia
4',6-Diamidino-2-Phenylindo, Dihydrochloride (DAPI)	Life Technologies Australia Pty. Ltd., Mulgrave, Australia
Acetic acid (glacial)	Merck Serono Australia Pty. Ltd., Frenchs Forest, Australia
AlexaFluor-594 Click-iT EdU cell proliferation Kit	Life Technologies Australia Pty. Ltd., Mulgrave, Australia
Antipain ($C_{27}H_{44}N_{10}O_6$)	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
sAPPα recombinant protein	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
B27 supplement	Life Technologies Australia Pty. Ltd., Mulgrave, Australia
Bio Rad DC protein assay kit	Bio Rad Laboratories Pty. Ltd., Gladesville, Australia
Bio Rad Silver Stain Plus Kit	Bio Rad Laboratories Pty. Ltd., Gladesville, Australia
cOmplete ultra protease inhibitor tablets	Roche Diagnostics Australia Pty. Ltd., Castle Hill, Australia
CNBr-activated Sepharose 4B	GE Healthcare Bioscience AB, Uppsala, Sweden
Chymostatin ($C_{31}H_{41}O_6N_7$)	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
CysC primer (Cst 3) primer	Qiagen Pty. Ltd., Chadstone, Australia
CysC (Recombinant mouse protein)	R&D Systems, Inc. Minneapolis, MN
CysC (Recombinant human protein)	My Biosource San Diego, CA, USA
DAKO mounting medium	DAKO Australia Pty. Ltd. Campellfield, Australia
Deoxyribonuclease-1	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Dulbecco's modified Eagle's medium (DMED)	Life Technologies Australia Pty. Ltd., Mulgrave, Australia
E-64 ($C_{15}H_{27}N_5O_5$)	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Fatty – acid free bovine serum albumin (BSA)	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia

Table 2.1 (Cont'd)

Material	**Supplier**
Fetal bovine serum (FBS)	Life Technologies Australia Pty. Ltd., Mulgrave, Australia
Glyceraldehyde-3-phosphate dehydrogenase GAPDH primer	Geneworks Pty. Ltd., Hindmarsh, Australia
Glycerol	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Glycine	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Human recombinant epidermal growth factor (EGF)	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Human recombinant basic fibroblast growth factor (bFGF)	PeproTech. Rocky Hill, USA
Hydrochloric Acid (37% w/v)	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Igepal CA-630	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Immobilon chemiluminescent substrate	Millipore Australia Pty. Ltd, Kilsyth, Australia
β -Mercaptoethanol	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Non-fat dry milk powder	Woolworths Ltd., Bella Vista, Australia
Normal goat immunoglobulin G	R&D Systems Inc. Minneapolis, MN
Normal mouse immunoglobulin G	Dako Australia Pty. Ltd. Campbellfield, Australia
Paraformaldehyde	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Penicillin /streptomycin	Life Technologies Australia Pty. Ltd., Mulgrave, Australia
Pepstatin A ($C_{34}H_{63}N_5O_9$)	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Poly-L-lysine	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Polyvinylidene fluoride membrane (PVDF)	Bio Rad Laboratories Pty. Ltd., Gladesville, Australia
Potassium Chloride (KCl)	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Potassium dihydrogen phosphate (KH_2PO_4)	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Protein G agarose gel	Roche Products Pty. Ltd., Dee Why, Australia
RNeasy mini Kit	Qiagen Pty. Ltd., Chadstone, Australia

Table 2.1 (Cont'd)

Material	**Supplier**
RT2 First Strand Kit	Qiagen Pty. Ltd., Chadstone, Australia
Silver Stain Plus	Bio Rad Laboratories Pty. Ltd., Gladesville, Australia
Sodium azide (NaN_3)	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Sodium biocarbonate ($NaHCO_3$)	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Sodium chloride (NaCl)	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Sodium deoxycholate	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Sodium phosphate monobasic dehydrate (Na_2HPO_4)	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
SYBR master mix	Qiagen Pty. Ltd., Chadstone, Australia
Synthetic human sequence Aβ peptides (>95% pure)	Keck Biotechnology Resource Laboratory, New Haven, CT
Tris-base	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Triton X-100	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia
Trypan Blue	Sigma-Aldrich Pty. Ltd. Castle Hill, Australia

Table 2.2 Cell culture media, buffers and solutions used in this study

Cell culture media	
NSPC Proliferation medium	Dulbecco's modified Eagle's medium (DMEM) contain 2% v/v B27 supplement, 20 ng/mL Human recombinant EGF and Human recombinant bFGF, 100 U/mL penicillin and 100 μg/mL streptomycin.
NSPC Differentiation medium	Dulbecco's modified Eagle's medium (DMEM) contain 2% v/v B27 supplement, 1% v/v heat inactive fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin.
Buffers and solutions	
Phosphate buffered saline (PBS)	137 mM NaCl, 2.7mM KCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.4
TBS-T (Immuno blotting)	150 mM NaCl, 25 mM Tris-base, pH 8.0, 0.05% Tween-20
SDS-PAGE running buffer	25 mM Tris-base, 192 mM Glysine, 0.1% SDS
Laemmli sample buffer	10% β-Mercaptoethanol, 2% SDS, 50 mM Tris pH 6.8, 10% glycerol
Cell lysis buffer	150 mM NaCl, 50 mM Tris, 0.5% w/v sodium-deoxycjolate, 1% v/v Igepal-CA630, 0.1% SDS, pH 7.4
Electroblotting buffer	25 mM Tris, 192 mM Glysine, 20% v/v Ethanol pH 8.4
Coupling buffer	0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3
Acetate buffer	0.1 M Acetate acid, 0.5 M NaCl, pH 4.0

Table 2.3 Primary and secondary antibodies used in this study

Primary antibodies			**Supplier**
Mouse anti human - Aβ monoclonal antibody (IgG) 6E10			Covance Pty. Ltd. North Ryde, New South Wales, Australia
Goat anti mouse IgG CysC antibody			R&D Systems Inc. Minneapolis, MN
Mouse anti IgG βIII tubulin monoclonal antibody			Promega Australia (Alexandria, Australia)
Rabbit anti IgM NG2 chondroitin sulfate proteoglycan antibody			Millipore Australia Pty. Ltd, Kilsyth, Australia
Mouse anti IgG GFAP monoclonal antibody			BD Bioscience North Ryde, New South Wales, Australia
Rabbit anti IgG Nestin polyclonal antibody			Millipore Australia Pty. Ltd, Kilsyth, Australia
Secondary antibody	**Conjugate**	**Application**	**Supplier**
Goat - anti mouse IgG	Alexa 594	ICC	Life Technologies Australia Pty. Ltd., Mulgrave, Australia
Goat - anti mouse IgG	Alexa 488	ICC	Life Technologies Australia Pty. Ltd., Mulgrave, Australia
Goat - anti rabbit IgM	Alexa 594	ICC	Life Technologies Australia Pty. Ltd., Mulgrave, Australia
Rabbit anti- Goat	HRP	WB	DAKO Australia Pty. Ltd. Campellfield, Australia
Goat anti - mouse	HRP	WB	DAKO Australia Pty. Ltd. Campellfield, Australia

Chapter 3

Role of CysC in amyloid precursor protein-induced proliferation of neural stem/progenitor cells

3.1 Introduction

NSPCs are self-renewing, multipotent cells that can produce all of the major cellular phenotypes in the nervous system (Reynolds & Weiss 1992) (Richards et al 1992). NSPCs are important, not only because they produce the entire complement of neuronal and glial cells of the mature nervous system and continue to generate new neurons throughout life, but also because they may be useful for the therapeutic replacement of cells in neurodegenerative diseases. The mechanisms that promote NSPC proliferation are only partially understood (Small et al 2001). Both EGF and bFGF are well studied for their roles in stimulating NSPC proliferation *in vitro* (Moyse et al 2008, Richards et al 1992). However, other autocrine growth factors produced by the NSPCs themselves may also be necessary for optimum growth.

APP is a 110–130 kDa integral type I transmembrane glycoprotein that has been extensively studied for its role in Alzheimer's disease (AD) (Small et al 2001). Despite the very large number of published studies on APP, the normal function of APP remains a mystery. APP is encoded by a single gene located on chromosome 21 (Patterson et al 1988). APP is post-translationally glycosylated and phosphorylated and can be cleaved by two major proteolytic pathways. In one pathway, sequential cleavage of APP by α- and γ-secretase generates a large ectodomain fragment (sAPPα), that is secreted into the extracellular milieu, and a small C-terminal fragment (the APP intracellular domain, or AICD), that may have a role in regulating gene expression. In the other pathway, cleavage of APP by β- and γ-secretase generates a different ectodomain fragment (sAPPβ), as well as the AICD peptide. Cleavage of APP via this second pathway also generates the Aβ that accumulates in AD (Nunan & Small 2000).

Although the normal function of APP is poorly understood, the pattern of APP expression suggests that it may be important for neuronal growth and differentiation, not only in the developing brain but also in the mature or aging nervous system. The expression of APP has been shown to increase as the nervous system matures (Small et al 1992). APP expression increases as NSPCs mature into neurons, and soluble APP has been reported to promote neural differentiation (Freude et al 2011, Lee & Cole 2007). APP may also play a role in later stages of neuronal development. For example, soluble APP is reported to stimulate neurite outgrowth in a variety of cell systems (Chasseigneaux et al 2011, Gakhar-Koppole et al 2008, Mattson et al 1992, Milward et al 1992, Small et al 1994). Studies have shown that APP expression is increased in the olfactory neuroepithelium at the developmental stage when neurogenesis and neurite outgrowth begin (Clarris et al 1995). Similarly, APP has been reported to regulate a number of developmental functions including neuronal migration (Young-Pearse et al 2007) and cell growth (Hornsten et al 2007, Joshi et al 2009). A role for APP in cell growth is supported by the rapid up-regulation of APP that occurs in response to axonal injury (Blumbergs et al 1995, Gentleman et al 1993, Itoh et al 2009). Dystrophic neurites found around amyloid plaques are highly immunoreactive for APP (Cochran et al 1991, Cras et al 1991, Tabaton et al 1992), consistent with the possibility that APP may play a role in neural repair.

Neurogenesis is reported to be increased in transgenic mice that overexpress APP. For example, Jin *et al.* (2004) reported a 2-fold increase in BrdU-labelled cells in PDGF-$APP_{sw,Ind}$ mice at 3 months of age. More recently, López-Toledano and Shelanski (2007) reported similar findings. The increase in neural precursor proliferation was attributed either to a compensatory mechanism resulting from disease pathology in the mice (Jin et al 2004a) or to a direct effect of Aβ (Lopez-Toledano & Shelanski 2007).

To address the role of APP in NSPC proliferation and neurogenesis, the growth and proliferation in culture of NSPCs derived from APP transgenic mice (Tg2576) and from APP knockout (APP-KO) mice was examined. This study showed that the proliferation rate of NSPCs from APP-overexpressing cells is increased and that the proliferation of NSPCs from APP-KO cells is decreased when compared with the corresponding background strain NSPCs. Furthermore, these studies showed that this effect is mediated by a secreted factor. Despite previous suggestions that sAPPα can influence the growth of neural stem cells (Freude et al 2011, Hayashi et al 1994, Lazarov & Demars 2012), no evidence was found that the effect on NSPCs is mediated by sAPPα. Instead, the studies showed that APP-induced NSPC proliferation is mediated, at least in part, by secreted CysC.

3.2 Materials and Methods:

3.2.1 All materials used in this chapter are shown in Table 2.1.

3.2.2 In vitro measurement of cell number and cell proliferation

Dissociated cells cultured adherently on poly-L-lysine-coated 96-well plates were incubated for various periods. Then the viable cell number was measured using the alamarBlue Assay (Chapter 2). Cell proliferation was monitored by an EdU incorporation assay (Chapter 2).

3.2.3 Effect of conditioned medium on NSPC proliferation

Conditioned medium was collected from neurosphere cultures that had been grown over a period of 7 days. To examine the effect of conditioned medium on NSPC proliferation, dissociated cells from neurospheres were cultured adherently on poly-L-

lysine-coated 96-well plates in 100 µl/well of proliferation medium. Sixteen hours after plating, 100 µl of conditioned medium or normal proliferation medium (control) was added, and the cells were incubated for 3 or 5 days, after which cell number was measured using the alamarBlue assay.

3.2.4 Determination of the levels of sAPPα and CysC

The level of sAPPα and CysC in conditioned medium was determined by immunoblotting. The volume per well of conditioned medium that was analyzed was adjusted so that it represented the same number of viable cells, as determined using the alamarBlue assay. Routinely, ~10–30 µl was loaded into each gel lane for analysis. For the analysis of intracellular CysC, cells were washed with PBS and then lysed in cell lysis buffer prior to analysis by immunoblotting (Chapter 2).

3.2.5 Immunodepletion of sAPPα, Aβ or CysC

For depletion of sAPPα, Aβ or CysC from conditioned medium, mAb 6E10, anti-mouse CysC antibody, or goat immunoglobulin G (35 µg) was incubated with 500 µl of protein G agarose gel (Roche Products Pty. Ltd., Dee Why, Australia) overnight at 4°C in 5 ml of PBS. The gel was then washed three times with 5 ml PBS, after which the gel was incubated with 5.5 ml of conditioned medium for 3 h at 4°C. Finally, the gel slurry containing conditioned medium was centrifuged (10,000 × *g*), and the resulting supernatant fraction was assayed by immunoblotting or used for cell proliferation experiments.

3.2.6 Real-time PCR

RNA was extracted from the neurospheres of $n = 6$ independent mouse cohorts using an RNeasy mini kit as described by the manufacturer. Each preparation of neurospheres contained ~106 cells. Six independent RNA extracts were obtained from

each neurosphere preparation. cDNA was obtained from 400 ng of RNA with an RT2 First Strand kit as described by the manufacturer. The CysC primers (Cst3) were from Qiagen, and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were from Geneworks. Glyceraldehyde-3-phosphate dehydrogenase GAPDH was used as an internal control. CysC (Cst3) and GAPDH primers were used at a concentration of 10 μm. All samples were diluted 1:10 and analyzed in triplicate. Standard curves for Cst3 and GAPDH with concentrations 1, 0.5, 0.25, and 0.125 μg were used to quantify Cst3 mRNA using SYBR master mix. The results were analyzed using a LightCycler 480 (Roche Diagnostics Australia Pty. Ltd., Castle Hill, Australia).

3.3 Results

3.3.1 Analysis of APP levels and proliferation of NSPCs derived from Tg2576 mice and background strain C57Bl/6 × SJL mice

The amount of APP immunoreactivity in neurosphere cultures was examined by western blotting to confirm that the level of APP were higher in the Tg2576 cultures than in the background strain (C57Bl/6xSJL) littermate control cultures. At 2, 4, and 6 days after plating, conditioned medium was analyzed for APP by immunoblotting with mAb 22C11, which recognizes both mouse and human APP and the APP homolog amyloid protein-like protein-2, and with mAb 6E10, which recognizes human sAPPα. The results confirmed that APP levels were much higher in the medium of Tg2576 neurosphere cultures than in the background strain cultures (Fig. 3.1, A). A major band of 100 – 110 kDa was detected in the medium, corresponding to the molecular mass of sAPPα.

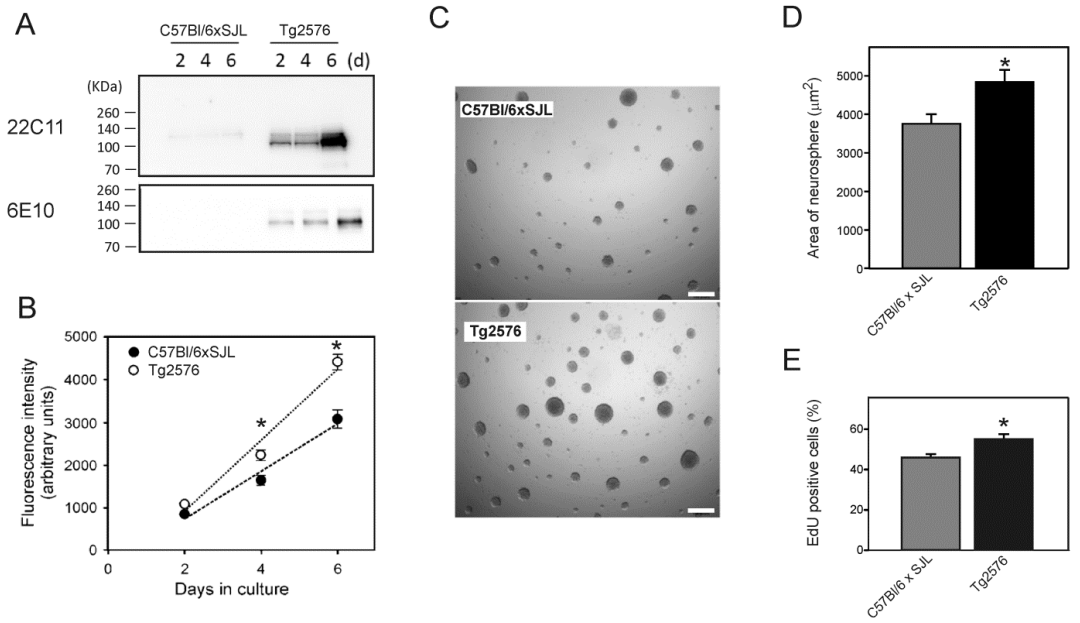

Figure 3.1 Analysis of APP levels and proliferation of NSPCs derived from Tg2576 mice and background strain C57Bl/6xSJL mice. A. Western blot analysis of the level of APP in the conditioned medium of neurosphere cultures. Blots were stained with mAb 22C11, which recognizes both human and mouse APP, and mAb 6E10, which is specific for human-sequence APP. B. Analysis of the proliferation of NSPCs. Neurospheres were dissociated and the isolated cells seeded on poly-L-lysine coated 96-well plates at a density of 2000 cells per well. After 2, 4 or 6 days in culture, the relative number of cells was estimated using the alamarBlue assay. Fluorescence intensity was taken as an index of cell number. Values are means ± SEM (n=3 wells). C. Phase-contrast microscopy of neurospheres after 7 days in culture. Scale bar = 200 mm. D. Quantitation of the area of neurospheres from experiment shown in C. Values are means ± SEM (n=200 neurospheres). E. EdU incorporation assay of cell proliferation. NSPCs were cultured in proliferation medium for 4 days prior to incubation for 8 h with EdU (30). The result shows that a greater proportion of Tg2576 NSPCs were actively proliferating (incorporating EdU) than the corresponding background strain cells. The graph shows the mean values (± SEM) of % cells incorporating EdU (n=30). * = significantly different from corresponding values for the background strain C57BL/6xSJL cells ($P<0.05$, experiment in panel B, one-way ANOVA with post-hoc Tukey's test; experiment in panel D, Student's t test).

Next, to examine the role of APP in NSPC proliferation, the proliferation of cells derived from Tg2576 mice was compared with that of the background strain controls. Neurosphere cultures were dissociated into a single cell suspension on day 7, and then cells were cultured adherently on poly-L-lysine-coated 96-well plates. The proliferation of the cells was measured using the alamarBlue assay. Fluorescence intensity in an alamarBlue assay was taken as an index of the number of viable cells. Overall, the growth rate of the cells derived from the Tg2576 mice was significantly greater ($p < 0.05$, one-way ANOVA with post hoc Tukey's test) than that of the cells derived from the background strain mice (Fig. 3.1, B).

The growth of neurospheres was also examined to determine whether the proliferation of NSPCs was increased in the Tg2576 cultures. Neurospheres were cultured for a period of 7 days, after which they were examined under phase-contrast microscopy (Fig. 3.1, C and D). Neurospheres derived from Tg2576 mice were on average greater in size than the neurospheres from the background strain mice, confirming that the growth of NSPCs in Tg2576 cultures was greater than that of the background strain cultures.

To confirm that the increased growth of the Tg2576 NSPC was due to a higher proliferation rate, proliferation was measured using an EdU uptake assay (Young et al 2013). The percentage of EdU-positive cells in the Tg2576 cultures was ~20% higher than the cultures derived from background strain mice (Fig. 3.1, E). These results clearly supported the view that the increased growth observed using the alamarBlue assay and in the neurosphere cultures was due to an increase in the amount of NSPC proliferation.

3.3.2 Analysis of proliferation of NSPCs derived from APP knockout mice

To examine whether expression of endogenous mouse APP influences NSPC proliferation and to rule out the possibility that the increase in proliferation observed in Tg2576 cultures was due to a factor unrelated to APP overexpression, the proliferation rate of NSPCs from APP KO mice was also examined. NSPC proliferation was decreased in APP-KO cultures when compared with the corresponding background strain (C57Bl/6) cultures (Fig. 3.2). The growth rate of the APP-KO cells, as assessed by an alamarBlue assay, was ~60% of that of the cells derived from background strain mice (Fig. 3.2, A). Furthermore, neurospheres from APP-KO mice were smaller and less numerous than the C57Bl/6 neurospheres (Fig. 3.2, B and C). In addition, the percentage of proliferating EdU-positive cells was also significantly decreased (Fig. 3.2, D) These experiments clearly demonstrated that endogenous APP was also involved in the regulation of NSPC proliferation.

3.3.3 Effect of neurosphere conditioned medium on NSPC proliferation

To determine whether the effect of APP on proliferation to determine whether the effect on proliferation was mediated by a factor that was secreted into the culture medium, conditioned medium was prepared over 7 days from Tg2576 and the corresponding background strain (C57Bl/6xSJL) neurosphere cultures. In parallel, dissociated C57Bl/6xSJL NSPCs were plated and incubated for 16 h. Conditioned medium from the neurosphere cultures, or unconditioned proliferation medium was then added to the dissociated cell cultures. The amount of proliferation was measured after 5 days of incubation. There was a higher rate of proliferation in cultures containing conditioned medium (whether from C57Bl/6xSJL or Tg2576 cells) than in cultures containing unconditioned medium (Fig. 3.3, A).

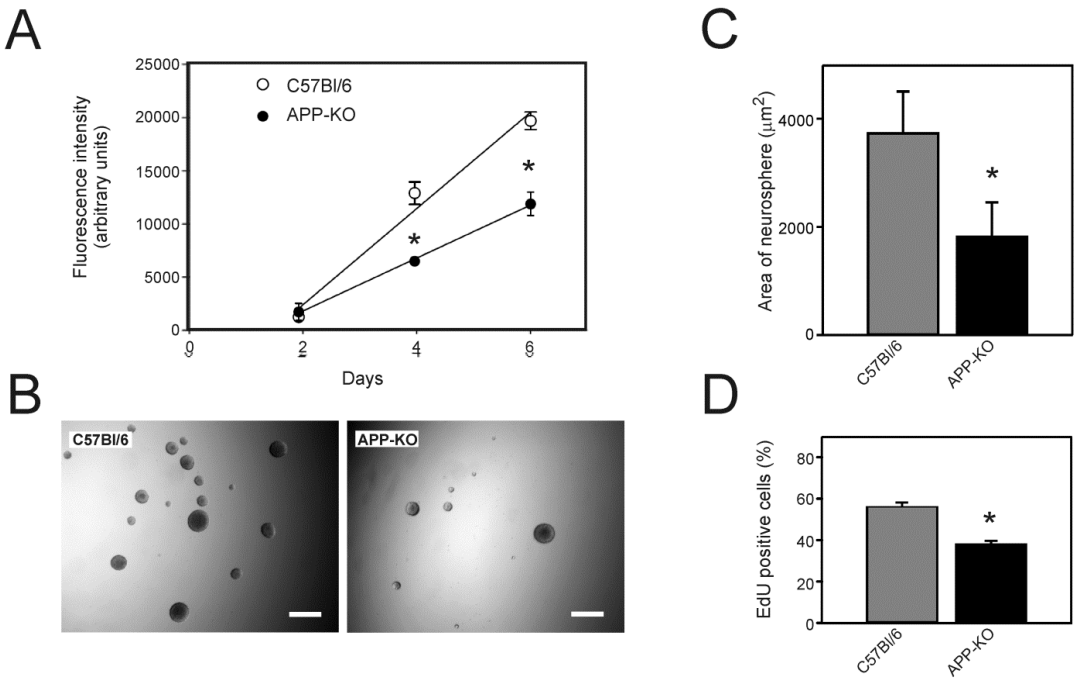

Figure 3.2 Analysis of NSPC proliferation derived from APP knockout and from C57Bl/6 (corresponding background strain) mice. A. Neurospheres were dissociated and the isolated cells seeded on poly-L-lysine-coated 96-well plates at a density of 2000 cells per well. After 2d, 4d or 6d in culture, the relative number of cells was estimated using the alamarBlue assay. Fluorescence intensity was taken as an index of cell number. Values are means ± SEM (n=3 wells). * = significantly different from corresponding values for the background strain C57Bl/6 cells ($P<0.05$, one-way ANOVA) B. Phase-contrast microscopy of neurospheres after 7d in culture. Scale bar = 200 mm. C. Quantitation of the area of neurospheres from the experiment shown in B. Values are means ± SEM (n=200 neurospheres). D. EdU incorporation assay of cell proliferation. The experiment was performed as described in Fig. 1. In panels C and D, the asterisk shows values that are significantly different from C57Bl/6 cultures ($P<0.05$, Student's t test).

Importantly, the conditioned medium from the Tg2576 cell cultures stimulated cell proliferation more than the conditioned medium from the C57Bl/6xSJL cultures, supporting the view that the increased proliferation in the Tg2576 cultures was due to the secretion of a factor into the conditioned medium.

To test this hypothesis further, the effect of conditioned medium from APP-KO cell cultures on NSPC proliferation was examined. This time, isolated NSPCs grown adherently were incubated with unconditioned medium (control), conditioned medium from APP-KO neurosphere cultures, or conditioned medium from the corresponding background strain C57Bl/6 neurosphere cultures (Fig. 3.3, B). In contrast to the results with Tg2576 cultures, the APP-KO conditioned medium was significantly less potent in stimulating proliferation than the background strain conditioned medium. This result again supported the view that there was a factor secreted into the medium of APP-expressing cells that increased cell proliferation.

3.3.4 Effect of sAPPα and Aβ peptides on NSPC proliferation

As it has been reported that sAPPα can stimulate neural stem cell proliferation or differentiation (Freude et al 2011, Hayashi et al 1994, Lazarov & Demars 2012), the effect of recombinant human sAPPα on proliferation in our cultures was examined. However, despite repeated experiments aimed at determining whether sAPPα can stimulate NSPC proliferation, it was not possible to demonstrate any effect of sAPPα over a range of different concentrations (50–2000 ng/ml) (Fig. 3.4, A). Despite suggestions that Aβ amyloid may stimulate stem cell proliferation (Lopez-Toledano & Shelanski 2007), it was not possible to find any effect of Aβ1–40 or Aβ1–42 on proliferation (Fig. 3.4, A).

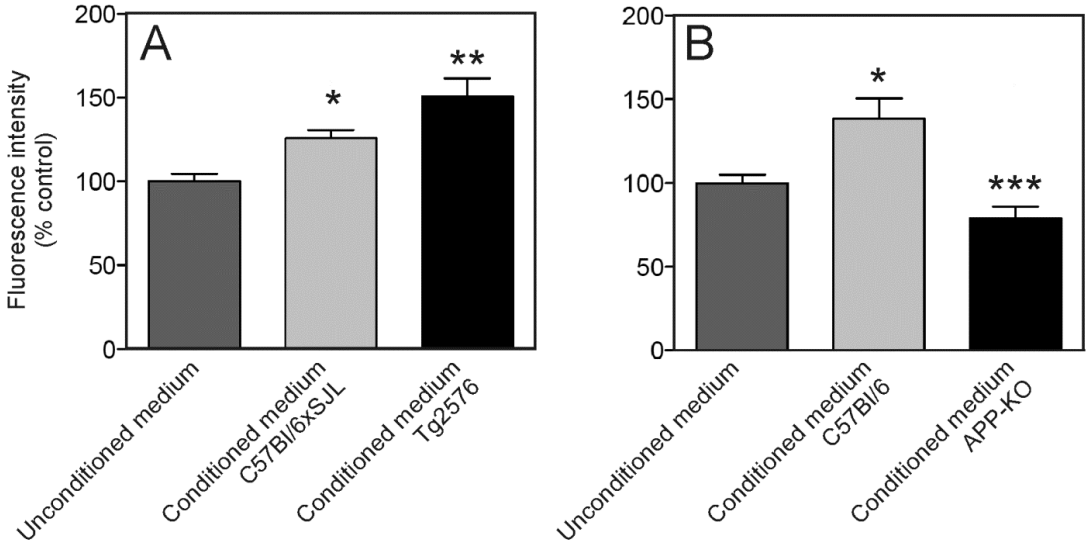

Figure 3.3 Effect of neurosphere conditioned medium on NSPC proliferation. A. Effect of unconditioned medium, conditioned medium from Tg2576 neurosphere cultures (CM-Tg2576) and conditioned medium from C57Bl/6xSJL neurosphere cultures (CM-C57Bl/6xSJL) on the growth of C57Bl/6xSJL NSPCs. Medium was conditioned for 7 d prior to adding to cultures to test for the effect on proliferation over a period of 5 d. Cell number is shown as a % of the fluorescence intensity measured in an alamarBlue assay compared to control (no conditioned medium). Values are means $\pm$ SEM. * = significantly different from unconditioned medium (control) ($P<0.05$). ** = significantly different from unconditioned (control) medium and from CM-C57Bl/6xSJL ($P<0.05$). B. Effect of unconditioned medium, conditioned medium from APP knockout neurosphere cultures (CM-APP-KO) and conditioned medium from C57Bl/6 neurosphere cultures (CM-C57Bl/6) on the growth of wild-type (C57Bl/6) NSPCs. Medium was conditioned for 7 d prior to adding to cultures to test for the effect on proliferation over a period of 5 d. Cell number is represented by the % of the fluorescence intensity measured in an alamarBlue assay compared with control (no conditioned medium). Values are means $\pm$ SEM. * = significantly different from unconditioned (control) medium ($P<0.05$). *** = significantly different from unconditioned (control) medium and from CM-C57Bl/6 ($P<0.05$).

To eliminate the possibility that the lack of effect of recombinant sAPPα might be due to the fact that the protein not being in a native conformation, the effect of removing endogenous sAPPα from the conditioned medium was tested (Fig. 3.4, B). More than 98% of the endogenously expressed sAPPα in the conditioned medium was removed by immunoabsorption with mAb 6E10 when compared with immunoabsorption with an immunoglobulin (Ig) control (Fig. 3.4, B *inset*). However, despite removal of most of the sAPPα, there was no decrease in the ability of the conditioned medium to stimulate proliferation. On the basis of these experiments, it was concluded that neither sAPPα nor Aβ (both of which are fragments of full-length APP that can be released into the culture medium) was the secreted factor that mediated the effect of APP overexpression on NSPC proliferation.

3.3.5 Levels of CysC and expression of CysC mRNA

As several studies have suggested that CysC is an important autocrine regulator of neural stem cell proliferation (Kato et al 2006, Taupin et al 2000), the possibility that CysC was the mediator of APP-induced NSPC proliferation was examined. Analysis of the conditioned medium from both Tg2576 cultures and APP-KO cultures by immunoblotting showed that CysC levels correlated with the effect on proliferation (Fig. 3.5). In addition to a major 14 kDa CysC band, a 16 kDa immunoreactive band was also present in the conditioned medium. This higher molecular mass band may represent a post-translationally modified form of CysC (Taupin et al 2000). Consistent with the view that the effect on proliferation was due to CysC, Immunoreactivity for CysC was found to be higher in the Tg2576 conditioned

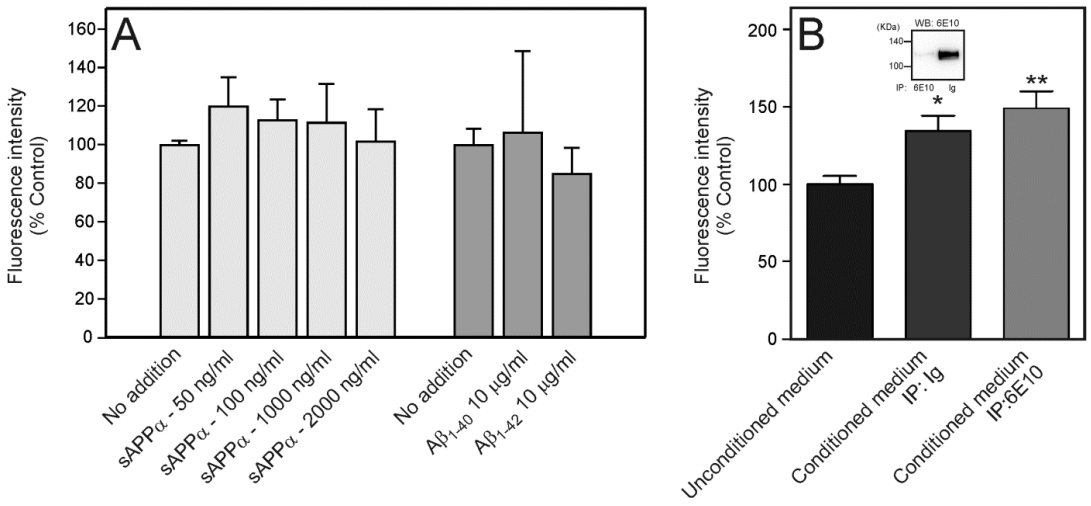

Figure 3.4 Effect of sAPPα and Aβ peptides on NSPC proliferation. Isolated NSPCs were prepared from C57Bl/6xSJL mice and cultured adherently on poly-L-lysine. Graphs shows cell number as represented by the % of the fluorescence intensity measured in an alamarBlue assay. Values are means ± SEM. A. Effect of recombinant sAPPα, $A\beta_{1-40}$ and $A\beta_{1-42}$ on NSPC proliferation. B. Immunoprecipitation of >98% of the sAPPa from Tg2576 conditioned medium did not decrease the ability of the conditioned medium to increase NSPC proliferation. The graph shows the effect of Tg2576 conditioned medium after immunoprecipitation with an immunoglobulin fraction (IP: Ig) and after immunoprecipitation with mAb 6E10 (IP: 6E10). Inset shows western blot analysis of the conditioned medium from both fractions. * = significantly different from control incubation. ** = significantly different from control incubation, but not significantly different from IP: Ig incubation.

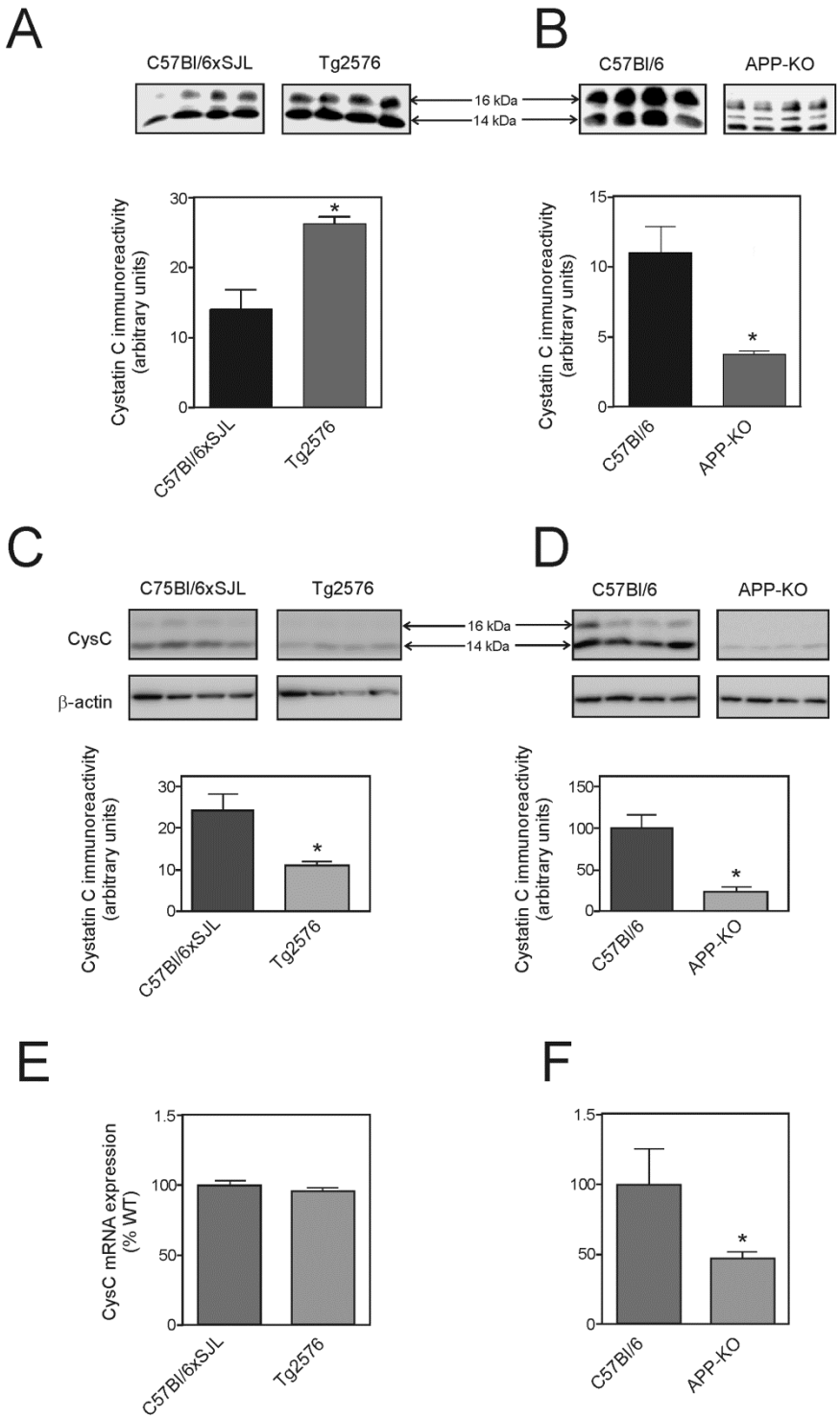
A
C57Bl/6xSJL
Tg2576
16 kDa
14 kDa
B
C57Bl/6
APP-KO
Cystatin C immunoreactivity (arbitrary units)
30
20
10
0
*
C57Bl/6xSJL
Tg2576
15
10
5
0
*
C57Bl/6
APP-KO
C
C75Bl/6xSJL
Tg2576
CysC
β-actin
16 kDa
14 kDa
D
C57Bl/6
APP-KO
Cystatin C immunoreactivity (arbitrary units)
30
20
10
0
*
C57Bl/6xSJL
Tg2576
150
100
50
0
*
C57Bl/6
APP-KO
E
CysC mRNA expression (% WT)
1.5
100
50
0
C57Bl/6xSJL
Tg2576
F
1.5
100
50
0
*
C57Bl/6
APP-KO

Figure 3.5 Levels of CysC and expression of *CysC* mRNA. Figure shows representative western blots and quantification of CysC immunoreactivity by image-capture analysis (panels A-D). Values are means ± SEM (n=4). A. Western blotting analysis of CysC in conditioned medium of Tg2576 mouse NSPC cultures and the corresponding background strain (C57Bl/6xSJL) cultures. Each lane represents a sample from a different line of NSPCs. B. Western blotting analysis of CysC in conditioned medium of APP-KO mouse NSPC cultures and the corresponding background strain (C57Bl/6) cultures. Each lane represents a sample from a different line of NSPCs. All statistical comparisons were made comparing staining intensities of images derived from the same blot. E & F. *CysC* mRNA expression determined by real-time PCR. Values are means±SEM (n=6). * = significantly different ($P<0.05$) from corresponding background strain control cultures (Student's t test).

medium (Fig. 3.5, A), and to be lower in the APP-KO cell conditioned medium (Fig. 3.5, B).

To determine whether the changes in secreted extracellular CysC reflected changes in the intracellular pool, the level of cell-associated CysC was examined (Fig. 3.5, C and D). Surprisingly, the level of CysC in the cell lysates was lower in both Tg2576 cultures and APP-KO cultures when compared with the corresponding background strain cells for each group. The level of *CysC* mRNA was also analyzed by real-time PCR (Fig. 3.5, E and F). These experiments showed that although the level of *CysC* mRNA expression was lower in the APP-KO cells, there was no significant difference in expression in the Tg2576 cells.

3.3.6 Role of secreted CysC in stimulating NSPC proliferation.

Similar to previous studies (Kato et al 2006, Taupin et al 2000), CysC increased NSPC proliferation in a concentration-dependent manner (50–100 ng/ml CysC) (Fig. 3.6, A). As the level of CysC in the conditioned medium of background strain cells was higher than this concentration range (Fig. 3.6, B panel i), this indicated that the concentration of CysC in the conditioned medium in both wild-type and background strain cells was sufficiently high to influence NSPC proliferation. Therefore, the possibility that the endogenous secreted factor in the conditioned medium was identical to CysC was tested. CysC was immunodepleted from the conditioned medium of both C57Bl/6xSJL and Tg2576 cultures (Fig. 3.6, B panel ii) and the effect of the immunodepleted medium on cell proliferation was measured.

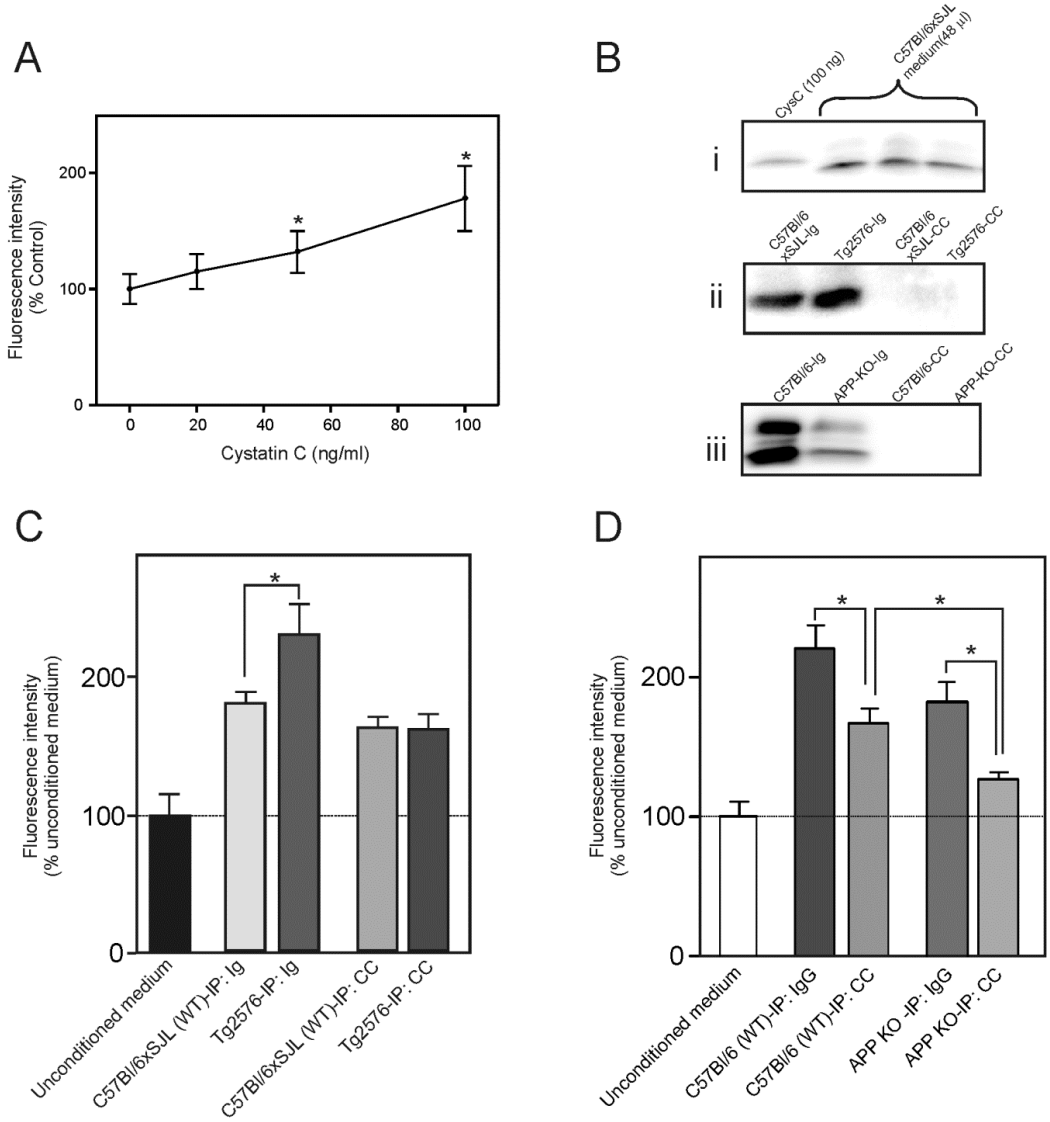
A
Fluorescence intensity
(% Control)
200
100
0
0
20
40
60
80
100
Cystatin C (ng/ml)
*
*
B
CysC (100 ng)
C57Bl/6xSJL
medium(48 µl)
i
C57Bl/6
xSJL-Ig
Tg2576-Ig
C57Bl/6
xSJL-CC
Tg2576-CC
ii
C57Bl/6-Ig
APP-KO-Ig
C57Bl/6-CC
APP-KO-CC
iii
C
Fluorescence intensity
(% unconditioned medium)
200
100
0
*
Unconditioned medium
C57Bl/6xSJL (WT)-IP: Ig
Tg2576-IP: Ig
C57Bl/6xSJL (WT)-IP: CC
Tg2576-IP: CC
D
Fluorescence intensity
(% unconditioned medium)
200
100
0
*
*
*
Unconditioned medium
C57Bl/6 (WT)-IP: IgG
C57Bl/6 (WT)-IP: CC
APP KO -IP: IgG
APP KO-IP: CC

Figure 3.6 Role of secreted CysC in stimulating NSPC proliferation. The number of viable cells was calculated from the fluorescence intensity in an alamarBlue assay. A. Effect of CysC on NSPC proliferation. Dissociated neurosphere-derived cells were plated on poly-L-lysine-coated plates and then incubated for 5 days in proliferation medium containing various concentrations of CysC. Values are means ± SEM, (n=4) and shown as % of the control value (no added CysC). * = significantly different from the control group (no CysC) ($P<0.05$, ANOVA with post-hoc Tukey's test). B. Western blots analysis of CysC immunoreactivity in cell culture medium. (i) Comparison of the level of CysC in 48 ml of C57Bl/6xSJL cell medium compared with 100 ng of recombinant CysC. (ii) Western blot stained for CysC showing efficiency of immunodepletion of CysC from the C57Bl/6xSJL and Tg2576 conditioned medium. Ig = medium after immunodepletion with control immunoglobulin; CC = medium after immunodepletion with CysC antibody. (iii) Western blot stained for Cys C showing efficiency of immunoprecipitation of CysC from the C57Bl/6 and APP-KO cell medium. C & D. Immunoprecipitation of CysC removes the APP-induced growth factor that stimulates proliferation. C. C57Bl/6xSJL neurosphere-derived cells were incubated with unconditioned medium or conditioned medium from C57Bl/6 x SJL cultures or Tg2576 cultures that was previously immunoabsorbed with a non-specific Ig fraction (IP: Ig) or with an anti-CysC antibody (IP: CC). D. APP-KO neurosphere-derived cells were incubated with unconditioned medium or conditioned medium from C57Bl/6 cultures or APP-KO cultures that was previously immunoabsorbed with a non-specific Ig fraction (IP: Ig) or with an anti-CysC antibody (IP: CC). Values in panels C and D are means ± SEM (n=4) and shown as % of the mean of the values for unconditioned medium. * = significantly different ($P<0.05$, ANOVA with post-hoc Tukey's test).

Immunodepletion of CysC from the conditioned medium completely removed the APP-associated increase in NSPC proliferation (Fig. 3.6, C). Proliferation was not significantly decreased when background strain NSPC conditioned medium was immunodepleted of CysC. However, after immunodepletion of CysC from the Tg2576 conditioned medium, the level of NSPC proliferation was significantly lower than the corresponding incubation in which the conditioned medium was preabsorbed with Ig.

Similar results were obtained in separate experiments using conditioned medium from C57Bl/6 and APP-KO cultures. In these experiments, the immunodepleted conditioned medium was tested on cultures of APP-KO cells (Fig. 3.6, D). Immunodepletion of CysC (Fig. 3.6, B panel iii) resulted in a significant decrease in proliferation of NSPCs when compared with the corresponding incubations in which the conditioned medium was preabsorbed with Ig. Taken together, these results clearly indicated that CysC was a major contributor to the ability of APP to increase NSPC proliferation.

3.4 Discussion

The result presented in this chapter demonstrate that APP expression regulates the proliferation of NSPCs and that this effect is mediated, at least in part, by an APP-stimulated increase in CysC secretion. The results increase our understanding of both the normal function of APP and the mechanisms involved in neural stem cell proliferation and differentiation.

The findings also provide an explanation for previous studies demonstrating that neural stem cell proliferation is increased in transgenic mice that overexpress APP (Jin et al 2004a, Lopez-Toledano & Shelanski 2007). Studies by Jin *et al.* (2004)

reported that neural stem cell proliferation was increased in PDGF-APPsw,Ind mice. López-Toledano and Shelanski (2006) confirmed and extended this observation. In both studies, no clear demonstration of the mechanism of increased proliferation was provided, although it was suggested that the stimulation of proliferation may have been due to Aβ accumulation or some aspect of associated Aβ pathology (Jin et al 2004a, Lopez-Toledano & Shelanski 2007). The present studies clearly support the view that NSPC proliferation is directly influenced by the expression of APP, although it is possible that a product of APP metabolism may be responsible for this effect. No direct evidence that this effect was influenced by sAPPα or Aβ peptides was found.

Interestingly, although neural stem cell proliferation is increased in APP-overexpressing mice, there is no obvious phenotype resulting from this increase. For example, prior to the onset of AD-type pathology, the brains of young APP transgenic mice appear relatively normal. Nevertheless, subtle abnormalities may be present in these mice. For example, overexpression of APP in transgenic mice is reported to lead to changes in synaptic density (Mucke et al 1994), and another study reports that APP transgenic mice exhibit persistent locomotor hyperactivity (Rodgers et al 2012). Whether these abnormalities are due to an increase in the number of specific neuronal populations is unclear. In the case of APP knockout mice, there are clear abnormalities, most notably agenesis of the corpus callosum (Muller et al 1994). However, whether this phenotype is due to a neural stem cell proliferation deficit is also unclear.

A surprising finding to emerge from these studies was that the effect of APP overexpression was not mediated through sAPPα. A large number of studies have

suggested that sAPPα has trophic properties on neural stem cells (Freude et al 2011, Hayashi et al 1994, Kwak et al 2006a, Lazarov & Demars 2012, Zhou et al 2011b). In the present study, recombinant human sAPPα had no effect on proliferation, nor did immunoprecipitation of endogenous secreted mouse sAPPα inhibit proliferation. Instead, immunoprecipitation of CysC from the culture medium was found to decrease the APP-induced stimulation, clearly demonstrating that some of the effect on proliferation was due to CysC. Moreover, CysC stimulated NSPC proliferation, and levels in the culture medium were correlated with APP expression, clearly supporting this idea. Although there may also be inhibitory factors that are secreted by NSPC that can affect cell proliferation, the study showed that almost all, if not all, of the APP – induced NSPC proliferation is mediated through secretion of CysC. Thus, if there were a secreted inhibitory factor, it would have to contribute only a very minor role.

Interestingly, in experiments with APP-KO cells, there was a significant residual effect of APP, even after removal of most of the CysC. CysC-immunodepleted C57Bl/6 conditioned medium was still significantly more potent in stimulating the proliferation of APP-KO cells than the corresponding immunodepleted APP-KO conditioned medium (Fig. 3.6, D). This suggests the possibility that there is an additional, as yet unidentified, secreted molecule that also contributes to APP-induced NSPC proliferation. This effect was not seen in experiments where the effect of conditioned medium on C57Bl6xSJL cells was tested (Fig. 3.6, C). The reason for this difference is unclear, but it could relate to the fact that the APP-KO cells may be more sensitive to this unidentified factor.

The mechanism of the APP-stimulated increase in CysC and NSPC proliferation is also not yet known. The experiments suggest that there may be two mechanisms involved. Real-time PCR experiments showed that *CysC* expression was decreased in APP-KO cells when compared with the corresponding background strain cells. As the extracellular domain of APP (*i.e.* sAPPα) was not found to stimulate proliferation, this suggests that the APP intracellular domain (AICD) may be involved in mediating this effect. Indeed, based on an analogy with the Notch intracellular domain (NICD), which is also released by γ-secretase (Guruharsha et al 2012), a number of studies suggest that the AICD may regulate gene expression (Pardossi-Piquard et al 2005). Whether AICD regulates the expression of CysC is not yet known and will require further studies.

However, CysC expression was not increased in Tg2576 cells when compared with the corresponding background strain cells. Indeed, a surprising finding was that although CysC secretion was higher in the Tg2576 conditioned medium (Fig. 3.5, A), levels were lower in the cell lysate (Fig. 3.5, C). This suggests the possibility that the higher levels of CysC in the Tg2576 culture medium may be due to an increased rate of CysC secretion with a concomitant decrease in intracellular CysC. The idea that increased APP may result in increased secretion is consistent with published studies. For example, another group reported that APP overexpression can increase vesicle exocytosis in PC12 cells (Lee et al 2008a). Furthermore, the cytoplasmic domain of APP can interact with proteins associated with synaptic vesicle release such as synaptotagmin-1 (Kohli et al 2012). Thus, it is possible that the cytoplasmic domain of APP may possess two different functions: 1) it may be translocated to the nucleus

to alter gene expression or 2) it may interact with proteins on the cytoplasmic leaflet of the plasma membrane to alter events such as exocytosis. The balance of these two functions could conceivably be regulated by the level of APP expression and the degree of saturation of binding to different adaptor proteins.

Finally, the study may have implications for understanding the role of CysC in the pathogenesis of AD. It is interesting to note that a polymorphism (G73A) in CysC has been linked to AD (Hua et al 2012). Furthermore, CysC is increased in regions around Aβ deposits in the AD brain (Kaur & Levy 2012, Steinhoff et al 2001), suggesting that it may play a role in pathogenesis or in response to Aβ pathology. Indeed, two groups found that CysC may have a protective effect as APP transgenic mice that had higher CysC expression were found to have diminished Aβ deposition (Kaeser et al 2007, Mi et al 2007). As APP is also increased in dystrophic neurites around amyloid plaques, it is tempting to speculate that increased CysC may be due to an increase in local APP expression. Further studies on the role of APP in regulating CysC expression in the AD brain may help to identify new targets for drug development in AD.

Chapter 4

Effect of cysteine protease inhibitors on NSPC proliferation

4.1 Introduction

CysC has been identified as an endogenous cysteine protease inhibitor (Bobek & Levine 1992). CysC is produced and released abundantly by a majority of tissues in mammals, and is found in high concentrations in CSF (Abrahamson et al 1986, Turk et al 2008). Recently, a potential role for cysteine proteases in NSPC proliferation has been proposed (Hu et al 2013, Kato et al 2006, Taupin et al 2000).

Cysteine proteases belong to one of five classes of proteases. These five groups include the aspartic, cysteine, metallo-, serine and threonine protease (Puente et al 2003). At present, three major families of cysteine proteases have been well studied in mammalian cells: caspases, calpains, and cathepsins. Caspases are important for programmed cell death (Fernando et al 2005, Nicholson & Thornberry 2003). However, caspase-3 activity has also been found to participate in cell development (Fernando et al 2005, Yoneyama et al 2014). Calpains are nonlysosomal proteases that, along with their endogenous inhibitor, calpastatin, participate in modulation of cell proliferation in the SVZ (Machado et al 2015, Santos et al 2012). Inhibition of calpains is also reported to increase NSPC proliferation (Machado et al 2015, Santos et al 2012). Cysteine cathepsins are secreted lysosomal peptidases that are suggested to be involved in NSPC development and differentiation (de Azevedo-Pereira et al 2011, Salvioli et al 2008). Activities of lysosomal cathepsins (Abrahamson 1994), caspases (Thornberry 1997) and cytosolic calpains (Turk et al 1997) may all be inhibited by members of the cystatin superfamily, including CysC.

Immunodepletion of CysC from APP overexpressing conditioned medium was found to prevent-APP induced NSPC proliferation (Chapter 3). Additionally, CysC has been

suggested to be an autocrine factor secreted by cells to promote NSPC proliferation in vitro (Dahl et al 2004, Taupin et al 2000). However, it still remains to be determined whether CysC acts like a growth factor (i.e binds to a growth factor receptor) or whether the effect on proliferation is mediated through inhibition of cysteine proteases.

To address this issue, NSPCs were treated with selective irreversible cysteine protease inhibitors (E-64, E-64C and E-64D), a reversible serine/cysteine protease inhibitor (antipain) an aspartic protease inhibitor (pepstatin A) as well as a broad spectrum protease inhibitor (chymostatin) to see whether other protease inhibitors could mimic the effect of CysC. The results showed that low concentrations of E-64 and antipain significantly promote NSPC proliferation in vitro, although NSPC growth was suppressed at high concentrations. An attempt was made to identify candidate cysteine proteases that might mediate the effect of CysC using affinity chromatography. However, no cysteine proteases were identified by the method. Nevertheless, the results still provide strong evidence that regulation of cysteine protease activities may affect NSPC proliferation.

4.2 Materials and methods

4.21 Materials

Stock solutions of protease inhibitors were prepared as follows. E-64 and antipain were dissolved in sterilized deionized water at a concentration of 30 mM and 50 mg/ml respectively, while E-64C, E-64D, pepstatin A and chymostatin were prepared in dimethyl sulfoxide (DMSO) at a concentration of 5 mg/ml, 10 mg/ml, 36 mM and 20 mM respectively. All the protease inhibitors were stored at -20°C prior to use.

4.2.2 NSPC proliferation

NSPCs were prepared as described in Chapter 2. Dissociated NSPCs were cultured adherently on poly-L-lysine coated 96-well plates at a density of 2000 cells per well in proliferation medium containing various concentrations of protease inhibitors (E-64, E-64C, E-64D, antipain, pepstain A and chymostatin). Cells were then incubated for 3 and 5 days. Cell number was measured using the alamarBlue assay (Chapter 2).

4.2.3 Preparation of CysC - Sepharose

To prepare a CysC - Sepharose affinity resin, recombinant CysC (1 mg) was dissolved in 3.3 ml of coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and then incubated with 170 mg of cyanogen bromide (CNBr)-activated Sepharose (prepared in 700 µl of coupling buffer) at room temperature for 2 hours. A control CNBr-activated Sepharose 4B resin was prepared by a similar method but was incubated with coupling buffer alone, lacking CysC. After formation of the covalent bond between CysC and the CNBr-activated Sepharose, the resin was centrifuged at 200g and washed with 4 ml coupling buffer 3 times to remove the excess unbound CysC. The resin was incubated with 0.2 M glycine-HCl solution (pH 8.0) for 2 hours to block any remaining active groups. The CysC-Sepharose was then washed four times with 0.1 M acetic acid solution containing 0.5 M sodium chloride (pH 4.0). Then the CysC-Sepharose resin was resuspended in 1ml of PBS and stored at 4°C for use. The control resin lacking CysC was treated by the same procedure as the CysC resin.

4.2.4 Affinity purification and analysis of fractions

For affinity purification, 500 µl of either CysC-Sepharose or control-Sepharose was incubated separately with 5 ml of NSPC-conditioned medium and unconditioned

medium respectively at 4°C overnight. The resins were washed three times with 5 ml of 1×PBS. An aliquot (200 µl) of the resin, for silver stain analysis, was resuspended in an equal volume of Laemmli sample buffer (10% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 50 mM Tris-HCl pH 6.8, 10% (v/v) glycerol) and heated at 95°C for 5 minutes. The supernatant fraction was collected and 60 µl/lane loaded onto a 12% glycine SDS-polyacrylamide gel for electrophoresis. After electrophoresis, the protein was then visualized in the gel using a Bio-Rad silver stain kit (Table 2.1). The gel was fixed in a solution containing a 40% methanol/10% acetic acid (v/v) solution for 30 min. After that, the gel was immersed in Bio-Rad oxidizer for 5 min and then washed with deionized water for 15 min. The gel was then stained with the Bio-Rad silver reagent for 10 min, followed by a quick rinse with deionized water. The gel was developed with the Bio-Rad developer solution for 30 seconds until a brown precipitate appeared. Finally, the gel was immersed in 5% acetic acid (v/v) for 15 min to stop the staining reaction.

4.3 Results

4.3.1 Effect of protease inhibitors on NSPC proliferation

In order to examine whether cysteine protease inhibitors can promote NSPC proliferation, NSPCs were incubated with various protease inhibitors. The irreversible cysteine protease inhibitor E-64, the cysteine and serine protease inhibitor, antipain, the aspartic protease inhibitor, pepstatin A, and the broad-spectrum protease inhibitor chymostatin were tested over a range of concentrations, as was cysC (Fig. 4.1, A). The concentration of CysC was chosen based on two previous publications (Kato et al 2006, Taupin et al 2000), while the concentrations of Antipain, E-64, Chymostatin, Pepstatin A E-64D and E-64 C were chosen based on those recommended by the

manufacturer (see Chapter 2). Cell proliferation was estimated by cell viability, determined using an alamarBlue assay.

CysC promoted NSPC proliferation more than 50% at a concentration of 6.6 nM although it had little effect at concentrations of 3.3 nM or 1.32 nM (Fig. 4.1, A). E-64 (0.1 μM) stimulated NSPC proliferation by nearly 70%, but inhibited NSPC proliferation at concentrations of 10 and 100 μM (Fig. 4.1, B). Antipain promoted NSPC proliferation by approximately 30% at concentrations of 0.15 μM and 1.5 μM, but it suppressed NSPC proliferation at a concentration of 150 μM (Fig. 4.1, B). The aspartic protease inhibitor, pepstatin A, and the broad-spectrum protease inhibitor, chymostatin did not stimulate NSPC proliferation at any concentration. However, both inhibitors decreased proliferation at a concentration of 10 and 100 μM (Fig. 4.1, B). The data demonstrate that only the cysteine protease inhibitors, E-64 and antipain promoted NSPC proliferation. However, this occurred only at lower concentrations.

To investigate the mechanism of E-64's effect on NSPC proliferation further, two synthetic analogues of E-64, E-64C and E-64D, were tested for their effects on NSPC culture. E-64C is a membrane-impermeable cysteine protease inhibitor and binds to cysteine proteases in a similar manner to E-64 (Matsumoto et al 1999). E-64D, is an ethyl ester of E-64C, and is a membrane permeable inhibitor that inhibits intracellular proteases such as calpains (McGowan et al 1989).

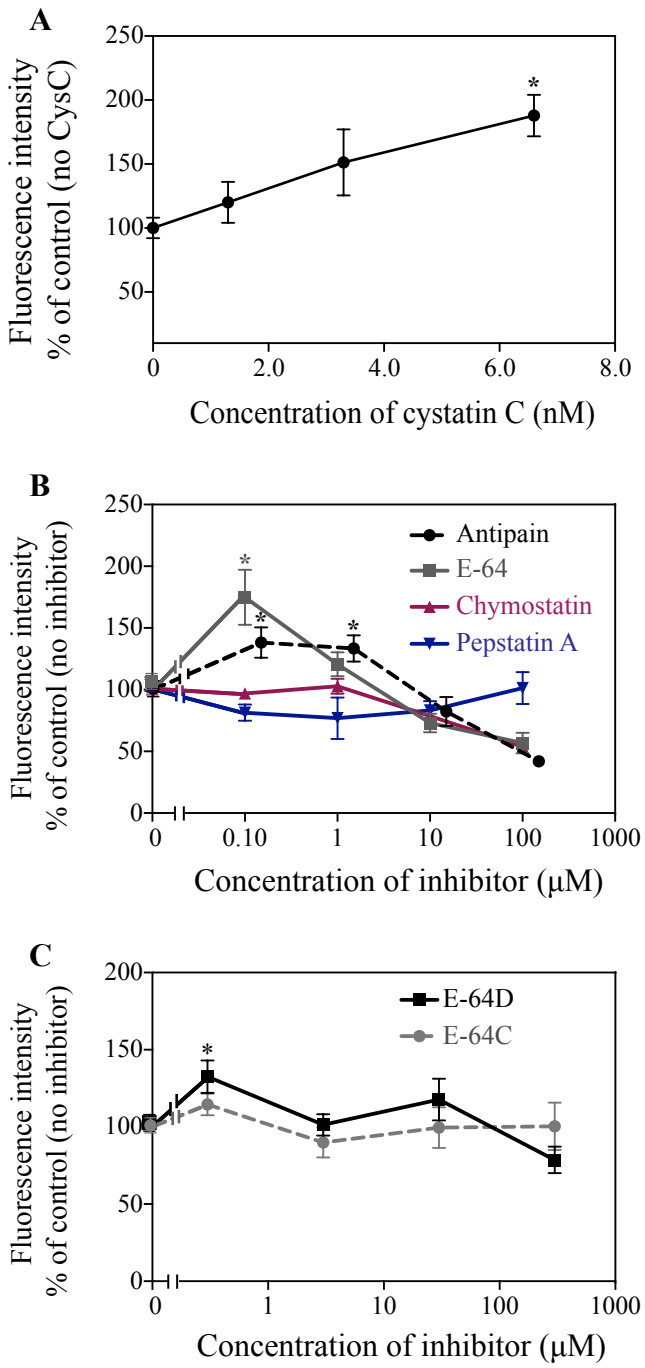

Figure 4.1 Effect of protease inhibitors on NSPC proliferation. Dissociated neurosphere-derived cells were plated on poly-L-lysine-coated plates at the density of 2000 cells per well and then incubated for 5 days in proliferation medium containing various concentrations of inhibitors.The relative number of cells was measured using the alamarBlue assay and fluorescence intensity shown in the figure was taken as an index of cell number. Values are mean ± SEM and expressed as % of the corresponding control (no inhibitors), ANOVA with post-hoc Tukey's test. Panel A: Effect of CysC on NSPC proliferation. * = significantly different from the control (no CysC) ($P < 0.05$, $n = 12$). Panel B: Effect of antipain, E-64, chymostatin and pepstatin A on NSPC proliferation. For antipain and E-64, * = significantly different from the control (no antipain or E-64) $P < 0.05$, $n = 6$; for chymostatin and pepstatin A, $P > 0.05$, $n = 6$. Panel C: Effect of E-64C and E-64D on NSPC proliferation. E-64C $P > 0.05$, $n = 10$, E-64D, * = significantly different from the conrresponding vehicle control $P < 0.05$, $n = 9$.

The results indicated that E-64D slightly promoted NSPC proliferation at a concentration of 0.3 μM, whereas E-64C did not affect NSPC proliferation at the same concentration (Fig. 4.1, C). Neither E-64C nor E-64D exerted an effect on NSPC proliferation at concentrations of 3, 30 and 300 μM (Fig. 4.1, C).

4.3.2 CysC affinity purification of proteases in conditioned medium

As CysC is cell membrane impermeable and yet it stimulated cell proliferation, it was logical to look for secreted proteases that might inhibit proliferation. Cell surface might also have been a possibility. However, a number of studies show that cell – surface proteases are generally also secreted (Lozzo 1998). In order to identify proteases that might bind to CysC, CysC affinity chromatography was performed on the NSPC conditioned medium. CysC-Sepharose was incubated with NSPC conditioned medium. The affinity resin was then collected and cysteine proteases bound to the resin were eluted and analyzed by protein electrophoresis. The bands of the proteins in the gel were visualized by a Bio-Rad silver stain kit. A resin lacking CysC was used as a control for comparison (Fig. 4.2). The study was aimed at identifying cysteine protease inhibitors that bind to proteases (Fig. 4.2). However, it was still uncertain whether the protease was a cathepsin (a secreted cysteine protease) or some other kind of protease. For this reason, cathepsin might not have been an ideal positive control.

A series of proteins were identified in both the unconditioned medium (lane 1) and conditioned medium (lane 2). Although a number of proteins were present in the unconditioned medium (presumably components of original medium), no specific

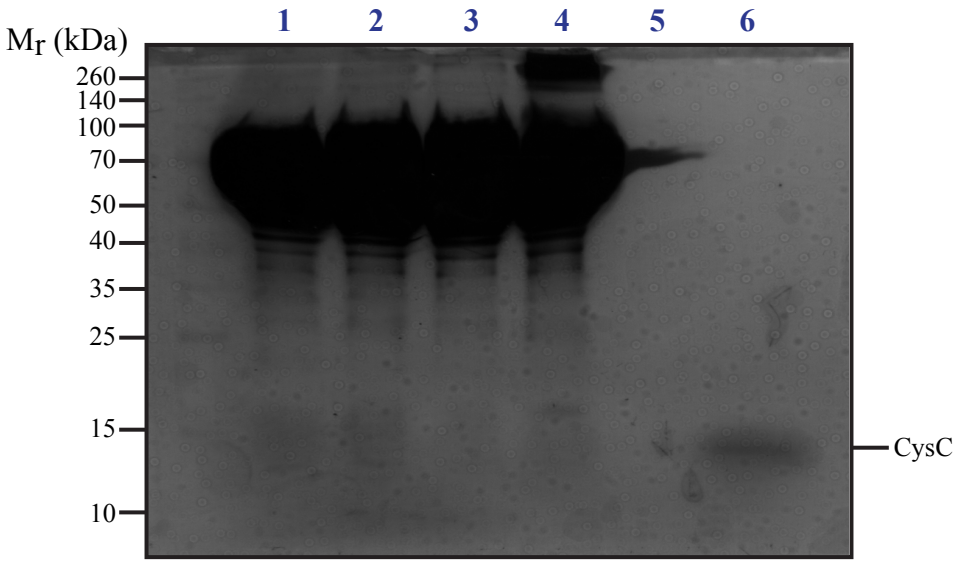

Figure 4.2 Analysis of fractions from cystatin C affinity chromatography by SDS - polyacrylamide electrophoresis. Proteins were separated by gel electrophoresis, and the proteins were visualized by silver staining. Lanes 1 to 6 were loaded with different fractions collected from cystatin C affinity chromatography. 1: Unconditioned medium. 2: Conditioned medium. 3: Conditioned medium after incubation with the control CNBr Sepharose. 4: Conditioned medium after incubation with the cystatin C Sepharose. 5: Eluate collected from conditioned medium eluted from control Sepharose. 6: Eluate collected from conditioned medium eluted from cystatin C Sepharose.

band was present in lane 2, that was not also present in lane 1. In addition, fractions containing conditioned medium that had been preadsorbed with control Sepharose (lane 3) and CysC Sepharose (lane 4) respectively also had a number of proteins. Nevertheless, no clear difference was seen between the two fractions. Lane 5 and 6 that were loaded with the bound fractions from the control CNBr Sepharose (lane 5) and CysC Sepharose (lane 6). No band was observed in the region around 21-30 kDa, which is the size most cysteine proteases are predicted to be. A broad band of approximately 13-15 kDa was observed in lane 6. This protein possesses a very similar molecular weight to that of CysC previously observed in NSPC culture (Hu et al 2013). Thus, this band was probably CysC that had leaked from the CysC Sepharose. This 13-15 kDa band was probably too small to be a cysteine protease, that normally have a molecular mass in the range of 21-30 kDa (Grzonka et al 2001).

In conclusion, no cysteine proteases were detected in the conditioned medium using silver staining. It is assumed that any cysteine proteases present in the conditioned medium are likely at too low a concentration for further analysis. As no specific proteins were detected by silver staining, this approach was not pursued.

4.4 Discussion

The results presented in this chapter showed that along with CysC, the specific cysteine protease inhibitors, E-64, E-64D and antipain can promote NSPC proliferation. However, neither the broad-spectrum protease inhibitor, chymostatin nor the aspartic protease inhibitor, pepstatin A, stimulated NSPC proliferation. Taken together, the results support the idea that NSPC proliferation is regulated by one or more cysteine proteases. Indeed, previous studies have shown that cysteine proteases

can ablate protein synthesis and thereby arrest cell proliferation (Chun et al 2002, Salmena et al 2003, Yadaiah et al 2013a, Yan et al 2001b), which also supports this view. On this basis, CysC may increase NSPC proliferation through acting as an inhibitor of cysteine protease activity.

Despite the plausibility of this hypothesis, no cysteine proteases were identified in the NSPC conditioned medium using the method of CysC-Sepharose affinity chromatography. Although CysC, E-64 and antipain stimulated NSPC proliferation at lower concentrations in this study (Fig. 4.1, A and B), they inhibit cysteine protease activities through different mechanisms. CysC binds adjacent to the catalytic center of cysteine proteases to block the substrate access, but without directly engaging with the active site of the cysteine protease (Rzychon et al 2004). E-64 and E-64D bind directly to the catalytic center of cysteine proteases (Feng et al 1996, Matsumoto et al 1999) by irreversibly modifying the thiol group of the cysteine protease to a thioether group. The inhibitory action of E-64 and E-64 D occurs through nucleophilic attack by the thiol group of the cysteine protease on the epoxide of the inhibitor (Hanada et al 1978a, Hanada et al 1978b, Matsumoto et al 1999, Tamai et al 1981). Importantly, E-64 does not interact with the functional thiol group of non-protease enzymes. The reversible cysteine/serine protease inhibitor, antipain also reversibly inhibits cysteine protease activities though the peptidyl aldehyde group binding covalently to cysteine proteases (Frommer et al 1979, Otto & Schirmeister 1997, Suda et al 1972). However, no matter whether these inhibitors affect the activity of cysteine protease directly or indirectly, inhibition of cysteine protease activities by CysC, E-64 and antipain, at least at low concentrations, led to increased NSPC proliferation (Fig. 4.1, A and B).

This argues that it is likely to be the cysteine protease activity itself that is important for the NSPC proliferation effect.

E-64, antipain and CysC are not able to cross the plasma membrane. Thus, it is likely that E-64 and CysC may affect NSPC proliferation by inhibiting an extracellular cysteine protease such as a cathepsin. Cathepsins are found to participate in protein degradation not only in the lysosome and secretory vesicles but also in the extracellular space (Green & Lund 2005, Hook et al 2012, Stoka et al 2005). Overproduction of cathepsins is usually accompanied by extracellular secretion of procathepsins that are processed or activated automatically once in acidic conditions or by extracellular proteases or polysaccharides (Beckman et al 2009, Mason & Massey 1992, Pungercar et al 2009, Vasiljeva et al 2005).

As cysteine cathepsins are proposed to have multiple biological roles including cell growth and death, as well as the production of biologically active peptides (Gopinath et al 2013, Goulet et al 2007, Hook et al 2012, Minokadeh et al 2010, Moles et al 2009, Nomura & Katunuma 2005, Veillard et al 2011), inhibitors that are able to block activation of procathepsin or to inhibit the activity of cysteine cathepsins may play a role in the regulation of cell proliferation. For example, a number of studies have indicated that cysteine cathepsins can degrade extracellular matrix (ECM) proteins to influence homeostasis of the ECM. Digestion of the ECM could exert an effect on NSPC proliferation (Gattazzo et al 2014, Hou et al 2003, Kurtz & Oh 2012, Maciewicz & Wotton 1991). For this reason, regulation of the activity of these extracellular cysteine cathepsins by inhibitors like E-64 or CysC may affect NSPC proliferation. As the serine protease inhibitor, antipain can increase NSPC

proliferation, and can also block activity of serine proteases, a role for serine proteases in NSPC proliferation cannot be ruled out (Xia et al 2013).

In this study, E-64 promoted NSPC proliferation, whereas two other synthetic analogues of E-64, namely E-64C and E-64D, exerted either a poor effect (E-64D) or no effect (E-64C) on NSPC proliferation (Fig. 4.1, C). Like E-64, E-64C is non-cell permeable, and preferentially affects activities of cysteine proteases in a similar way to E-64 (Matsumoto et al 1999, Matsumoto et al 1989). The possible explanation for the different effects of the E-64 analogues may be that alteration of the structure of E-64 may affect the ability of the compound to undertake nucleophilic attack on the epoxide (Matsumoto et al 1999, Matsumoto et al 1989). While E-64C and E-64D are known to inhibit a number of cysteine proteases in a manner similar to E-64, it is possible that the proteases responsible for NSPC proliferation may not be readily inhibited by the analogues.

E-64D is an ethyl ester form of E-64C and is cell membrane permeable. E-64D was found to slightly promote NSPC proliferation at a concentration of 0.3μM (Fig. 4.1, C). E-64D also contains the epoxide structure that is involved in the interaction within the active site of the cysteine protease in E-64 (Hanada et al 1978a, Hanada et al 1978b, Matsumoto et al 1989, Wilcox & Mason 1992). The reason why E-64D stimulated NSPC proliferation, but E-64C did not, is not clear. However, one possibility is that alterating the molecular structure of E-64C to E-64D promotes an interaction between the inhibitor and the cysteine proteases. Therefore, E-64D may stimulate NSPC proliferation by acting on an extracellular cysteine protease (Hook et al 2011), as was observed with CysC, antipain and E64. Alternatively, addition of an

ethyl group onto E-64C increases the lipophilicity of the compound, which making E64D membrane permeable and capable of being transported into the cells. Thus it might be argued that this is an evidence for an action on an intracellular protease. If this was the case, it would be surprising to find that CysC, a membrane -impermeable inhibitor would also be active. It is more likely that the ethyl esterification of E-64C to E-64D affects the compound's affinity for the proteases. Or it may act by another undefined mechanism.

In this study, low concentrations of E-64, E-64D and antipain exhibited a stimulatory effect on NSPC proliferation while higher concentrations usually exerted an inhibitory effect on cell proliferation. CysC has also been reported to promote NSPC proliferation in a concentration-dependent manner, as high concentrations of CysC reportedly suppress NSPC proliferation (Taupin et al 2000). Therefore, low concentrations of cysteine protease inhibitors may block the activities of the cysteine protease that is responsible for NSPC proliferation. High concentrations of cysteine proteases inhibitors are more likely tend to excessively suppress cysteine proteases activities required for NSPC proliferation or maintenance (Kurtz & Oh 2012).

CysC affinity chromatography was used to further investigate which cysteine proteases might interact with CysC to regulate NSPC proliferation. However, no cysteine proteases were identified in this study. No proteins were identified in the region of 21-30 kDa in which many cysteine proteases are normally found. A band was clearly observed around 13-15 kDa in the lane 6 (Fig. 4.2) at a position corresponding to CysC. This band had the same molecular weight as CysC previously identified in NSPC conditioned medium (Chapter 3). This band was probably a

contaminant that had leaked from Sepharose resin and therefore it was not analyzed further. However, it cannot be concluded that CysC does not bind to a cysteine protease from this result. Firstly, the amount of extracellular cysteine proteases in the NSPC conditioned medium that interact with CysC may be too low to be characterized by silver staining. CysC may possibly bind to several cysteine proteases which are present at very low concentrations in the NSPC conditioned medium. Secondly, it is possible that the cysteine protease may bind to CysC but may not bind well to CysC-Sepharose. This could be due to Sepharose resin attaching to CysC close to the binding site for cysteine proteases. This possibility is quite likely given the fact that CysC is a relatively small protein. Unfortunately, there was not sufficient time to investigate these possibilities further.

Chapter 5

Role of APP in neural stem/progenitor cell differentiation

5.1 Introduction

Neural stem or progenitor cells (NSPCs) are able to self-renew and also retain the ability to give rise to the major cell types of the CNS including glial and neuronal lineages (Palmer et al 1999, Reynolds & Weiss 1992, Richards et al 1992). The investigation of NSPC biology is important not only because NSPC persist into adulthood, but also because of the possibility that NSPCs may be a therapeutic agent for the treatment of neurodegenerative diseases or other neurological disorders via replacement of lost, or injured cells (Hsieh 2012, Taylor et al 2013). Theoretically, NSPCs may provide an inexhaustible cell source for cell repair or tissue engineering in neurobiology (Gil-Perotin et al 2013). However, the process of NSPC differentiation is controlled and modulated by a variety of endogenous factors in vivo (Huang et al 2010, Katsimpardi et al 2014, Lopez-Juarez et al 2012, Roybon et al 2009a). Thus a good understanding of how NSPC behaviour is regulated is crucial if NSPCs are to be used as a source of neural cell types that can function properly integrate into the existing circuitry.

β-Amyloid protein (Aβ) plays a central role in AD pathogenesis (Nunan & Small 2000, Zheng & Koo 2011). Aβ is produced by processing of the β-amyloid precursor protein (APP). However, the normal biological function of APP still remains elusive. Increased APP overexpression associated with neuronal differentiation has been observed during neuronal development (Clarris et al 1995, Hung & Selkoe 1994, Trapp & Hauer 1994). APP was reported to induce neural differentiation of pluripotent stem cells (Khandekar et al 2012). In addition, enhanced neuronal differentiation was also observed in APP overexpressing transgenic mice (Jin et al 2004a, Lopez-Toledano & Shelanski 2007, Yu et al 2009). However, studies on the

effect of APP deficiency found that embryonic stem cells from APP triple knockout mice still can differentiate into neurons both in vivo and vitro, indicating that APP expression does not play an essential role in the initiation of neuronal differentiation (Bergmans et al 2010). Nevertheless, delayed neuronal differentiation and development are also associated with decreased APP, APLP1 and APLP2 expression (Shariati et al 2013). Moreover, the soluble secreted fragments of APP, sAPPα and Aβ have been reported to drive NSPC differentiation, either towards an astrocytic or neuronal fate in vitro (Baratchi et al 2012, Chen & Dong 2009, Gakhar-Koppole et al 2008, Kwak et al 2006a, Lopez-Toledano & Shelanski 2007). Taken together, these findings suggest a role for APP in NSPC differentiation.

The study reported in Chapter 3 showed that NSPCs derived from APP transgenic mice (Tg2576) proliferate more rapidly than the corresponding wild type mice, while NSPCs obtained from APP KO mice proliferate less readily than their background strain of wild type mice (Hu et al 2013). The enhanced NSPC proliferation was induced by APP-mediated secretion of a protease inhibitor, CysC (Hu et al 2013). Neither Aβ nor sAPPα exerted an effect on proliferation.

To address the role of APP in NSPC differentiation, the differentiation of NSPCs derived from APP transgenic mice (Tg2576) and from APP knockout (APP KO) mice was examined. These studies showed that APP induces neuronal and astrocytic differentiation of NSPCs, but has no effect on oligodendrocyte differentiation. Although previous studies have suggested that sAPPα or Aβ induce NSPC differentiation (Heo et al 2007, Kwak et al 2006a, Lopez-Toledano & Shelanski 2007), in the present study, neuronal and astrocytic differentiation were not found to

be influenced by sAPPα or synthetic Aβ. Furthermore, CysC also did not induce NSPC differentiation.

5.2 Materials and methods

5.2.1 Materials used in this study are shown in Table 2.1.

5.2.2 NSPC differentiation

Neurospheres were prepared from Tg2576 mice and the corresponding background strain mice C57Bl/6×SJL, as well as APP KO mice and the corresponding background strain littermate mice C57Bl/6. NSPC cultures were prepared following the procedure described in chapter 3 section 3.2.1. After 7 days in culture, neurospheres were mechanically dissociated and the isolated cells were plated on poly- L- lysine coated coverslips in 24 -well plates at the density of 100,000 cells per well in proliferation medium (Table 2.2). The proliferation medium was removed after the cells were seeded on the coverslip, and then the cells were grown in a differentiation medium [(DMEM supplemented with 2% (v/v) B27, 100 units/ml penicillin, 100 units/ml streptomycin and 1% (v/v) heat - inactivated foetal calf serum (FCS)] and incubated for 5 or 14 days at 37°C in an atmosphere containing 5% CO_2. To test the effect of CysC, sAPPα, $Aβ_{40}$ and $Aβ_{42}$ on NSPC differentiation, cells were maintained in differentiation medium containing CysC, sAPPα, $Aβ_{40}$ and $Aβ_{42}$ for 5 days.

5.2.3 Immunocytochemistry

Cells were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, after which cells were permeabilized with 0.03% (v/v) Triton X-100 in PBS for 5 minutes, and then incubated in 10% (v/v) sheep serum in PBS for 1 hour to block non-specific binding sites. Fixed, permeablized cells were double immunostained with a mouse IgG anti-βIII tubulin antibody (1:1000 diluted in 10% (v/v) sheep serum in PBS) and a rabbit IgG anti-nestin antibody (1:5000 diluted in 10% (v/v) sheep serum in PBS), or a mouse IgG anti-GFAP antibody (1:3000 dilution in 10% (v/v) sheep serum in PBS) and a rabbit IgG anti-nestin antibody (1:5000 diluted in 10% (v/v) sheep serum in PBS) at 4°C overnight, respectively. After that the cells were incubated with a goat anti-mouse IgG conjugated to Alexa Fluor 488 or 594 (1:1000 diluted in 10% (v/v) sheep serum in PBS) or with goat anti-rabbit IgG conjugated to Alexa Fluor 488 or 594 (1:1000 diluted in 10% (v/v) sheep serum in PBS), and counterstained with 4', 6-diamidino-2-phenylindole DAPI at 1:10,000 dilution in PBS.

There was no need to permeablize the fixed cells that were immunostained with a rabbit IgM anti-NG_2 antibody. In this case, the fixed cells were directly incubated in 10% (v/v) sheep serum in PBS for 1 hour to block non-specific binding sites and then incubated in rabbit anti-NG_2 in 10% (v/v) sheep serum in PBS at 4°C overnight. After that the cells were incubated with a rabbit goat anti-IgM conjugated to Alexa Fluor 594 (1:1000 diluted in 10% (v/v) sheep serum in PBS), then the cells were permeablized with 0.03% (v/v) Triton X-100 in PBS for 5 minutes. The cells were blocked in 10% (v/v) sheep serum in PBS for 1 hour, and incubated with rabbit IgG anti-nestin antibody (1:5000 diluted in 10% (v/v) sheep serum in PBS) at 4°C

overnight. Finally, the cells were incubated with goat anti-rabbit IgG conjugated to Alexa Fluor 488 (1:1000 diluted in 10% (v/v) sheep serum in PBS) and counterstained with DAPI (at 1:10,000 dilution in PBS). The cells were mounted in DAKO mounting medium and allowed to dry overnight before imaging.

5.2.4 Image capture and data analysis

Images of fluorescently labelled cells were captured under the 20x objective using a Nikon Ti-E microscope (Nikon corporation, Tokyo, Japan). To quantify the amount of βIII tubulin$^+$, GFAP$^+$, and NG_2^+ fluorescence relative to DAPI$^+$ fluorescence, 9 random fields were selected from 1 coverslip and 3 coverslips were analyzed for each group in every experiment. There were approximately 100 cells in each field. Images were analysed using ImageJ version 1.46 software and statistical analysis was performed with GraphPad Prism version 5.04 software. Data were tested by Student's *t* test or one-way ANOVA. Post hoc comparisons were analyzed using Tukey's test. Differences were considered statistically significant when the probability (p) of the null hypothesis < 0.05. Data values are shown as the means ± S.E. All results were derived from at least three independent experiments in which cells were derived from at least three different mice of the same strain.

5.3 Results

5.3.1 Differentiation of NSPC

Previous in vitro studies on NSPC proliferation found that NSPCs derived from APP KO mice proliferated more slowly than NSPCs obtained from wild type (WT) mice of the background strain (C57BL/6), while NSPCs from APP overexpressing (Tg2576)

mice proliferated more rapidly than NSPC from corresponding wild type (C57BL/6 × SJL) mice (Hu et al 2013). This result showed that APP expression exerts an effect on NSPC proliferation.

To examine the possibility that APP also influences NSPC differentiation, the neuronal differentiation of NSPCs prepared from postnatal day 0 APP KO and the corresponding WT (C57BL/6) mice, along with Tg2576 mice and the corresponding background strain WT (C57BL/6 × SJL) mice was examined. After 5 or 14 days in culture, the cells were fixed and labelled with neuronal, astrocytic and oligodendrocyte progenitor (OPC) markers, β III tubulin, GFAP and NG2, respectively.

The proportion of β III tubulin$^+$, GFAP$^+$ and NG2$^+$ positive cells to total viable cells in the culture was determined. Three independent experiments were carried out and 21 fields were randomly selected from three separate cultures from each experiment with approximately 100 viable cells in each field.

The experiment showed that a relatively smaller percentage of APP KO NSPCs were stained with β III tubulin$^+$ than WT NSPCs after both 5 and 14 days in culture (Fig. 5.1, A and D). In contrast, there was a higher proportion of β III tubulin$^+$ neurons in the Tg2576 NSPC cultures than in the WT NSPC culture after 5 and 14 days (Fig. 5.1, A and D). The percentage of GFAP$^+$ cells in the Tg2576 NSPC cultures was similar to the percentage of GFAP$^+$ astrocytes in the WT culture after 5 days (Fig. 5.1, B and E). However, after 14 days, the proportion of GFAP$^+$ cells in the Tg2576 NSPC cultures was significantly higher than the percentage of cells with GFAP positive staining in

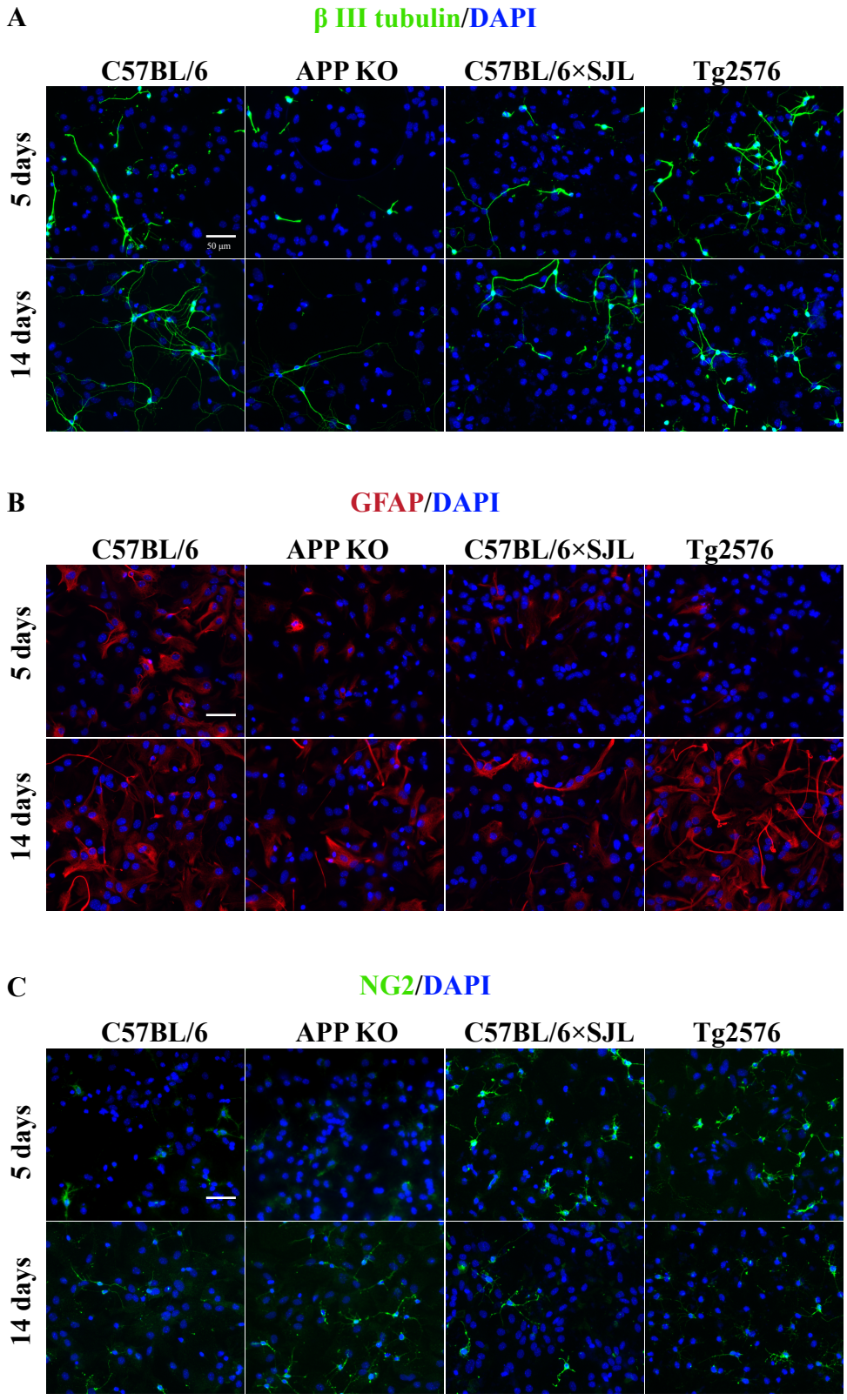

Figure 5.1 Effect of APP on NSPC differentiation. Neuronal, astrocytic and oligodendrocyte progenitor (OPC) differentiation was detected by expression of their specific marker. NSPCs were grown on poly-L-lysine coated coverslips in differentiation medium, and maintained for 5 or 14 days respectively before immunostaining for β III tubulin (panel A, neuronal marker), GFAP (panle B, astrocytic marker), or NG2 (panel C, oligodendrocyte progenitor marker).

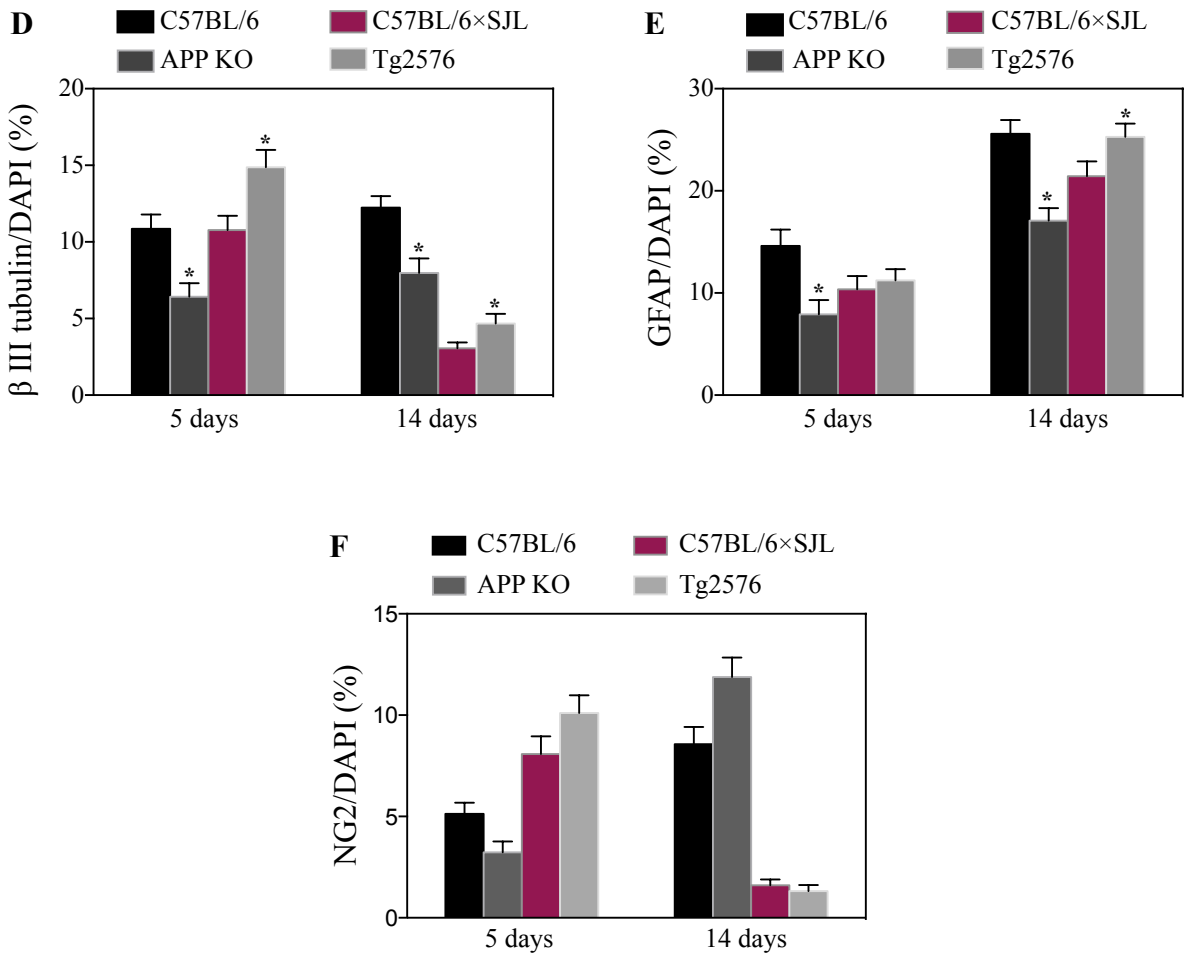

Figure 5.1 (Cont'd) The percentage of cells with β III tubulin, GFAP or NG2 expression was calculated by counting the number of cells with β III tubulin, GFAP and NG2 positive staining and dividing by the total number of cells stained with DAPI, respectively. Panels D - F show quantitation of immunofluorescence staining (A - C). The data were collected from three independent experiments, values are means. Two tailed t tests: panel D, C57BL/6 versus APP KO : $P < 0.001$, $n = 45$ (5 days) and $P < 0.001$, $n = 62$ (14 days); C57BL/6×SJL versus Tg2576, $P < 0.01$, $n = 61$ (5 days) and $P < 0.05$, $n = 58$ (14 days); Panel E: C57BL/6 versus APP KO, $P < 0.001$, $n = 40$ (5 days) and $P < 0.0001$, $n = 60$ (14 days); C57BL/6×SJL versus Tg2576, $P > 0.05$, $n = 63$ (5 days); $P < 0.05$, $n = 63$ (14 days); Two way ANOVA: Panel F: C57BL/6 versus APP KO, $P < 0.05$, $n = 60$ (5 days) and $P < 0.05$, $n = 59$ (14 days); C57BL/6×SJL versus Tg2576, $P > 0.05$, $n = 55$ (5 days) and $P > 0.05$, $n = 59$ (14 days).

the WT cultures (Fig. 5.1, B and E). A lower percentage of $GFAP^+$ cells was observed in APP KO NSPC cultures compared to the WT culture both after 5 days and 14 days (Fig. 5.1, B and E).

The percentage of $NG2^+$ cells in WT cultures was higher than in the APP KO cultures after 5 days, but after 14 days the proportion of $NG2^+$ cells was lower in the WT culture than in the APP KO culture after 14 days (Fig. 5.1, C and F). However, there was no difference in the proportion of $NG2^+$ cells between the Tg2576 and the WT cultures after 5 or 14 days.

5.3.2 CysC is not involved in APP induced differentiation

The studies reported in Chapter 2 showed that APP-induced NSPC proliferation was due to secretion of CysC. For this reason, the possibility that CysC is a mediator of APP-induced NSPC differentiation was examined. NSPCs from APP KO and the corresponding background strain WT (C57BL/6) mice were prepared and incubated in differentiation medium containing 0, 100 or 200 ng/ml of CysC. After 5 days, there was no difference in the proportion of β III $tubulin^+$ neurons and $GFAP^+$ astrocytes in any of the treatment groups (Fig. 5.2, A-F). This indicated that CysC did not influence NSPC differentiation into neurons or astrocytes and it suggested that the effects of APP on NSPC differentiation were not due to CysC secretion.

5.3.3 sAPPα, $Aβ_{40}$ or $Aβ_{42}$ do not influence neuronal and astrocytic differentiation

As NSPC differentiation was not influenced by CysC, the next question was whether differentiation was influenced by sAPPα, or by $Aβ_{40}$ or $Aβ_{42}$ which are the major secreted fragments of APP. Indeed, a few studies have reported sAPPα or Aβ may

exert a neurotrophic effect on neural stem cell differentiation (Chen & Dong 2009, Demars et al 2011, Fonseca et al 2013, Freude et al 2011). In order to explore the effect of sAPPα, $A\beta_{40}$ and $A\beta_{42}$ on NSPC differentiation, APP KO NSPCs were grown in differentiation medium containing sAPPα at a concentration of 10 nM which was previously reported to stimulate NSPC proliferation (Demars et al 2011), or $A\beta_{40}$ or $A\beta_{42}$ at a concentration of 1 μM which has been suggested to promote neuronal or astrocytic differentiation (Chen & Dong 2009). Controls for the corresponding vehicle were also included. The proportion of cells with β III tubulin$^+$ neurons, GFAP$^+$ astrocytes and NG2$^+$ OPCs (Fig. 5.3, A - C) was then calculated. However, after 5 days there was no difference observed in the percentage of cells positive for β III tubulin, GFAP and NG2 after treatment with sAPPα (Fig. 5.3, C and F) $A\beta_{40}$ (Fig. 5.3, A and D) or $A\beta_{42}$ (Fig. 5.3, B and E). It was concluded that the effect of APP on NSPC neuronal or astrocytic differentiation was probably not due to sAPPα, $A\beta_{40}$ or $A\beta_{42}$.

5.4 Discussion

The study reported in this chapter shows that APP can influence NSPC differentiation into neurons and astrocytes but plays little role in oligodendrocyte (OPC) differentiation. However, the data suggest that APP-induced neuronal and astrocytic differentiation is not due to secretion of CysC, nor due to the proteolytic products of APP processing, sAPPα, and $A\beta_{40}$ or $A\beta_{42}$.

Increased APP expression has been reported during neuronal differentiation (Hung et al 1992), which suggests a potential role for APP in neuronal fate development. Additionally, enhanced neuronal differentiation has been reported in young transgenic

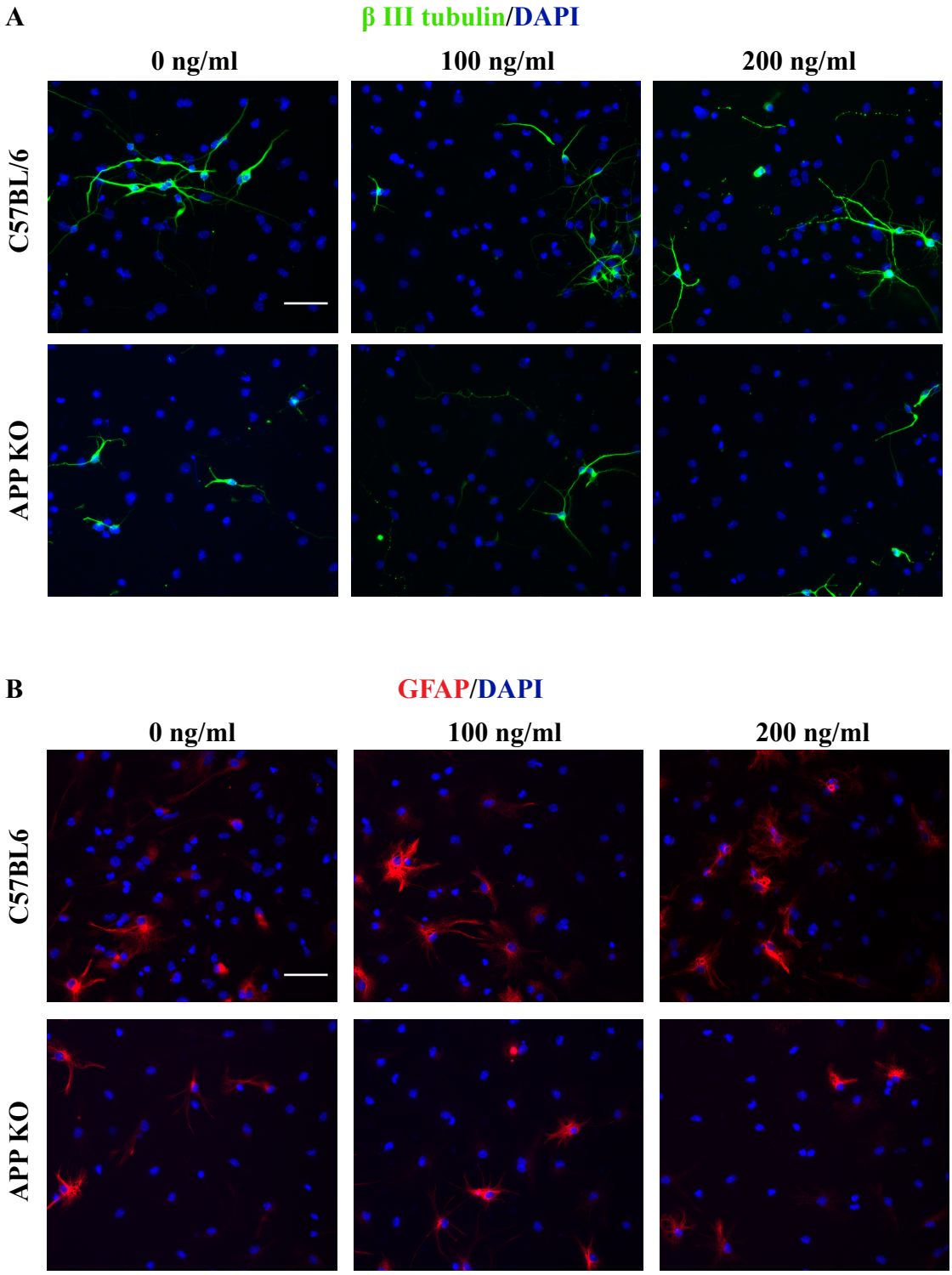

Figure 5.2 Effect of cystatin C on neuronal and astrocytic differentiation. NSPCs were grown in differentiation medium containing cystatin C for 5 days and then the cells were fixed and labelled for specific markers of neurons (β III tubulin) and astrocytes (GFAP). Panel A and B show representative immunofluorescence images.

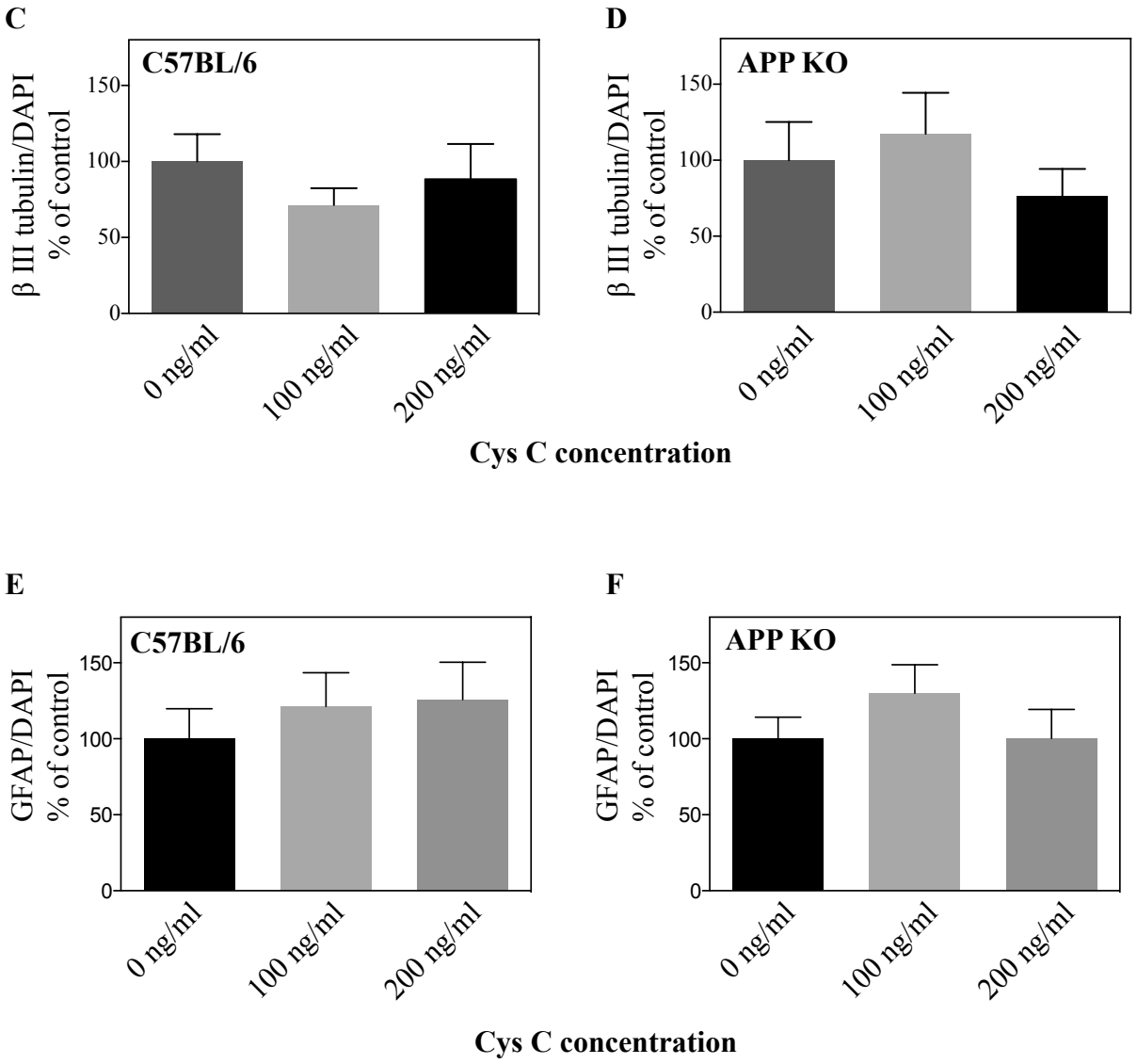

Figure 5.2 (Cont'd) Panel C - F show quantitation of immunofluorescence staining (A and B). The percentage of β III tubulin and GFAP expression in culture was determined as a ratio of the number of β III tubulin (panel A, green) or GFAP - positive cells (panel B, red) to the total number of cells stained with DAPI. In panels C, D, E and F, values are means ± SEM and shown as % of the control (0 ng/ml cystatin C) ($P > 0.05$, ANOVA with post-hoc Tukey's test).

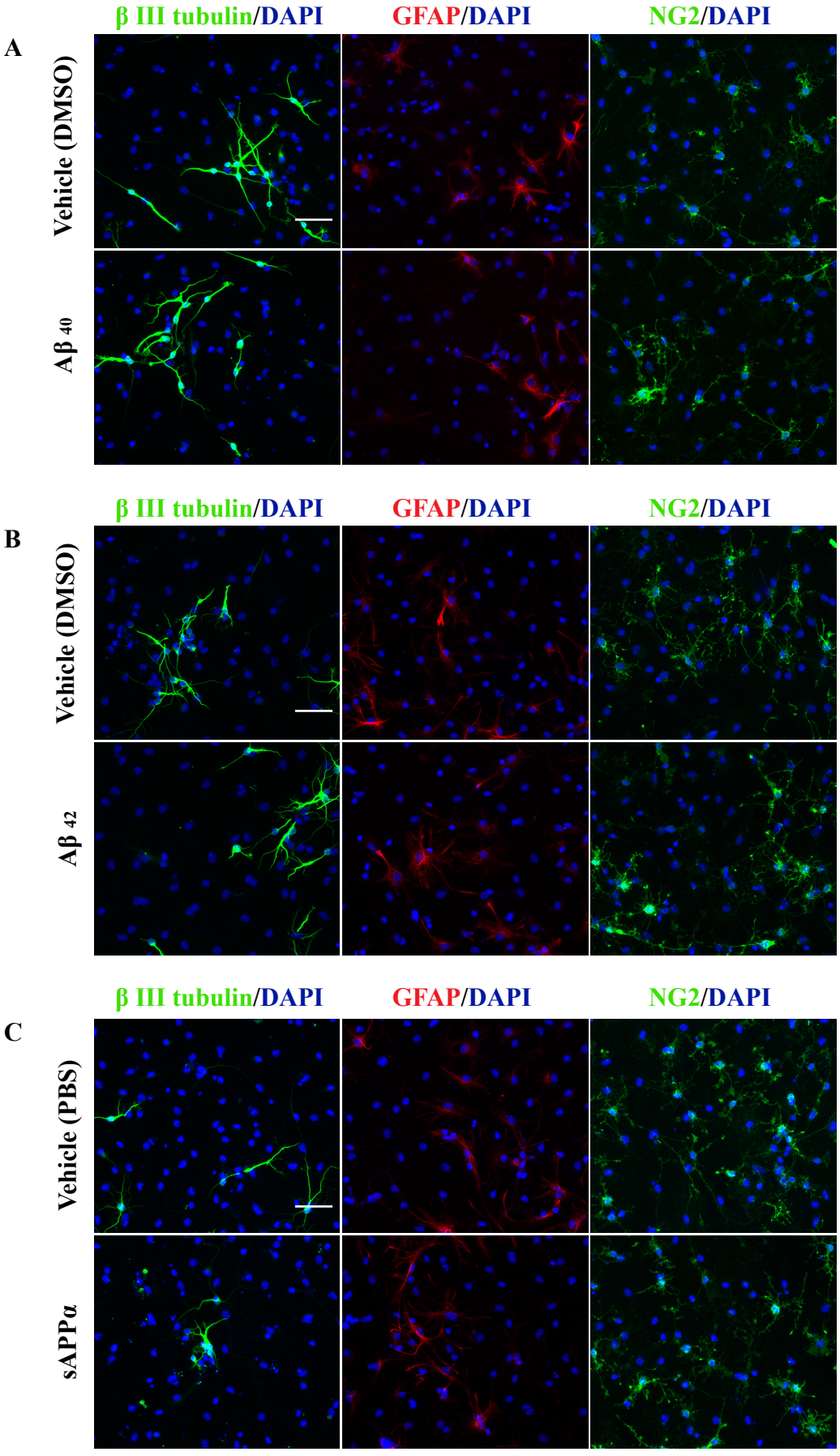

Figure 5.3 Effect of Aβ40, Aβ42 and sAPPα on NSPC differentiation. NSPCs from APP KO mice were differentiated for 5 days in culture and then treated with Aβ40 (1μM), Aβ42 (1μM) or sAPPα (100 nM). The cells were fixed and labelled for markers of neurons (β III tubulin), astrocytes (GFAP) and OPCs (NG2), respectively. Cells were counterstained with DAPI.

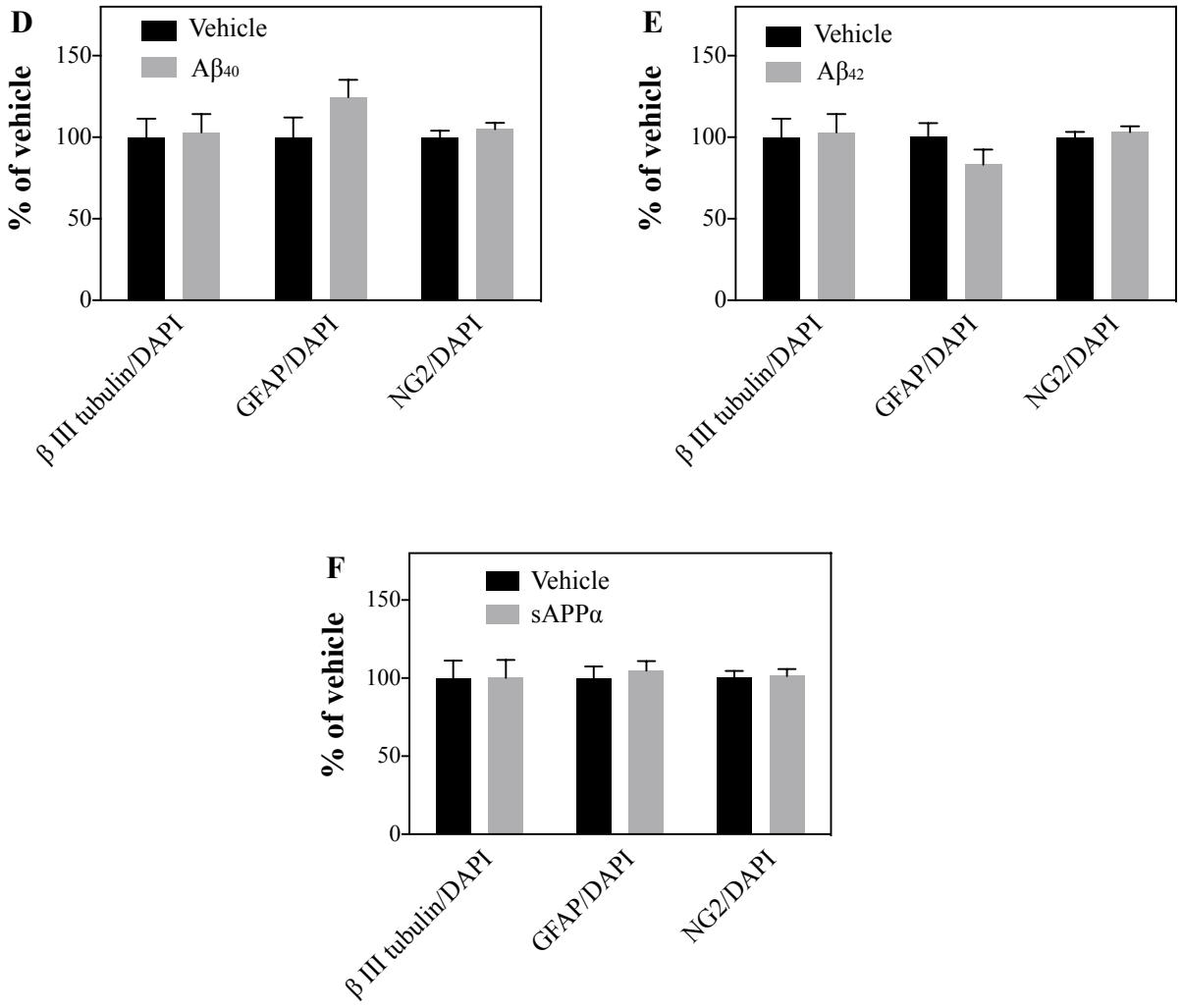

Figure 5.3 (Cont'd) Panel D - F show quantitation of immunofluorescence staining in panels A - C. The proportion of cells with β III tubulin (green, panel A), GFAP (red, panel B), or NG2 (green, panel C) staining was calculated as a ratio of the number of cells labelled with DAPI, respectively. Panels D, E and F show the effect of Aβ40, Aβ42 and sAPPα respectively versus their corresponding vehicle controls.Values are means ± SEM and shown as % of the corresponding vehicle control ($P > 0.05$, $n = 63$).

mice overexpressing APP (Lopez-Toledano & Shelanski 2007), a result consistent with the view that expression of APP may participate in neuronal differentiation (Fig. 5.1, A and D).

A few studies have reported that APP-induced multipotent NSPC differentiation is due to the major soluble form of APP, sAPPα (Demars et al 2011, Freude et al 2011, Kwak et al 2006a). However, this result was not observed in the current study, which found that recombinant sAPPα had no effect on NSPC differentiation (Fig. 5.2, C and F). Nonetheless, Kwak et al (2006) reported that the secreted form of APP (e.g sAPPα) can enhance glial differentiation but not neuronal differentiation in vivo. However, in the present study, NSPCs treated with sAPPα did not display increased astrocytic or OPC differentiation. The reasons for the difference are unclear. sAPPα may exert different effects in vitro and in vivo. Indeed, an in vivo situation is a much more complicated system than one an in vitro. Therefore the observed phenomena, such as enhanced glial differentiation in vivo, could be attributed to a series of complex signal pathways, not just to the infusion of sAPPα. An alternative reason is that cells in the different studies did not come from the same sources, and sAPPα may exert different effects on different types of neural stem cells (Freude et al 2011). Only further studies will be able to address this issue.

While this study found that APP causes neural differentiation, this was not due to Aβ production, because the two major forms of Aβ did not affect NSPC differentiation. Several studies have reported that Aβ peptides have neurotrophic effects and that they can stimulate NSPC differentiation (Chen & Dong 2009, Fonseca et al 2013, Haughey et al 2002, Lopez-Toledano & Shelanski 2004, Lopez-Toledano & Shelanski 2007,

Sotthibundhu et al 2009). Nonetheless, other groups reported the opposite results, i.e. that Aβ has an inhibitory effect on neurogenesis, neural differentiation or NSPC fate determination (Haughey et al 2002, He et al 2013). Once again, the reason for the difference between these studies is unclear. However, there are various forms of $A\beta_{40}$ and $A\beta_{42}$ including different aggregated forms of Aβ. It is possible that the forms of Aβ used in the present study were not the same as the forms that induced differentiation in the literature (Chen & Tang 2006, He et al 2013). For instance, He et al (2013) found that $A\beta_{42}$ oligomers induce NSPC senescence in adult hippocampus, while another two groups observed an increase in NSPC differentiation following $A\beta_{40}$ or $A\beta_{42}$ peptide treatment (Chen & Dong 2009, Fonseca et al 2013). Methods by which Aβ is prepared such as the solvent that is used to dissolve Aβ may lead to changes in Aβ conformation (Soto et al 1994, Zagorski & Barrow 1992). Aβ contains a completely hydrophobic C-terminal region that promotes β strand structure, and an N-terminal domain, that allows formation of different secondary structures (Soto et al 1994). However, the N-terminal domain of Aβ can exist either in an α helical or in β strand conformation based on the environmental conditions (pH, hydrophobicity of surrounding molecules). Therefore altered Aβ confirmation may also influence NSPC behavior (Chen & Dong 2009, Soto et al 1994, Sotthibundhu et al 2009).

Another possibility that may explain why there are differences about the effect of Aβ on NSPC differentiation is that the studies reported here were carried out on NSPCs derived from a different model. The response of these NSPCs may be variable depending on the system used. One study based using animal models reported a stimulatory effect of Aβ on neuronal differentiation of NSPCs in the SVZ

(Sotthibundhu et al 2009), while another study examining the effect of Aβ on NSPCs from the SVZ showed an inhibitory effect on neuronal differentiation (Haughey et al 2002). The reason for the discrepancy is unclear. To conclude, further studies on the effect of Aβ on NSPCs behaviour are required.

CysC has been reported to promote NSPC proliferation (Dahl et al 2003, Taupin et al 2000) and NSPC proliferation is regulated by APP through CysC (Hu et al 2013). A few papers have been reported that CysC can influence NSPC differentiation (Kato et al 2006, Kumada et al 2004). In one, CysC induced ES cells to differentiate into NSPCs and β III tubulin^{+} cells in this study (Kato et al 2006). CysC can also activate the GFAP promoter and induce astro-glial differentiation during mouse brain development (Kumada et al 2004). However, the result of this study did not support the idea that CysC can influence adult NSPC differentiation. It is possible that CysC may affect the development of ES cells into neural stem cells, as reported previously (Kato et al 2006), but it may have no effect on adult NSPC development and differentiation.

The molecular basis by which APP induces differentiation remains unclear and needs for future investigation. Studies done by the author in collaboration with Dr Marta Bolos found that an increased expression of a aproneural basic helix-loop-helix (bHLH) transcription factor, neurogenin 2 (Ngn 2) is likely to be a mediator of this effect (Bolos et al 2014). In that study, transfection of Ngn2 into APP KO NSPCs stimulated increased neuronal differentiation of NSPCs, whereas knocking down Ngn2 expression in WT NSPCs led to a decrease in neuronal differentiation (Bolos et al 2014). Therefore, APP overexpression may cause up-regulation of Ngn2 expression and thereby induce neuronal differentiation.

Ngn2 is unlikely to be involved in glial differentiation because neurogenins including Ngn2 are reported to promote neuronal differentiation, but inhibit glial differentiation (Sun et al 2001). APP may mediate glial differentiation via a BMP family signalling pathway (Kwak et al 2014), monocyte chemoattractant protein-1 expression (Vrotsos et al 2009) or interleukin-6 cytokine release (Kwak et al 2006b, Ringheim et al 1998). However, the mechanism by which APP modulates Ngn2 expression and NSPC differentiation remains unclear.

Chapter 6 Discussion

The neuropathology of AD was first described by Alois Alzheimer in 1907 (Alzheimer 1907). Despite intensive studies, the mechanism of AD development still remains unclear. There are no effective treatments to prevent, or even delay the progression of AD. AD is characterized by the deposition of amyloid plaques and neurofibrillary tangles in the brain (Kidd 1964, Terry 1963). Aβ is the major component of the amyloid plaques (Masters et al 1985), and is proposed to be the key causative agent of AD (Hardy & Selkoe 2002, Small et al 1992). A chronic imbalance of production and clearance of Aβ has been proposed to trigger progression of AD (Hardy & Selkoe 2002).

Aβ is produced by sequential proteolytic cleavage of the β - amyloid precursor protein (APP) (Kang et al 1987). According to the amyloid hypothesis of AD, several therapeutic approaches have proposed that are mainly aimed at reducing Aβ production via alteration of APP processing. Indeed, many compounds that target APP processing to lower Aβ production have already been studied in clinical trials (Small et al 2004). However, the biological function of APP is still unknown. A major motivation for this study was to understand the function of APP. APP has been intensively studied for its relationship with AD. Many second generation AD therapies are focusing on decreasing Aβ production through inhibition of APP proteolytic processing. Therefore, a better understanding of the function of APP is essential to develop approaches for AD treatment, as it is possible that altering APP metabolism may also alter its function and thereby induce toxicity.

Several studies have reported enhanced NSPC proliferation in APP overexpressing transgenic mice (Jin et al 2004a, Lopez-Toledano & Shelanski 2007, Yu et al 2009) as

well as in the brain of patients with AD (Jin et al 2004c). In addition, increased APP expression has been observed at early stages of neuronal differentiation, maturation and fate specification during embryonic development (Clarris et al 1995, Salbaum & Ruddle 1994). However, other studies have reported disrupted NSPC proliferation or differentiation in APP transgenic mice (Haughey et al 2002, Mu & Gage 2011). Several cytokines that are released during inflammation in the AD brain have been found to cause a deficit in NSPC proliferation or differentiation (Keohane et al 2010, Kohman & Rhodes 2013, Monje et al 2003). Thus, there is a possibility that the disease-related inflammation in mice that overexpress APP, and which produce Aβ, may be the cause of the altered neurogenesis in these studies.

It was hypothesized that APP is involved in the regulation of adult NSPC proliferation and differentiation. The studies presented in this thesis were aimed at exploring the potential role of APP in NSPC proliferation and differentiation by examining NPSC proliferation and differentiation in APP overexpressing and APP knockout NSPC culture and by further investigating the mechanisms involved in this action. Neurogenesis occurs not only in development but also persists into adulthood with active NSPCs continuously undergoing proliferation and differentiation in neurogenic niches (Alvarez-Buylla & Garcia-Verdugo 2002, Eriksson et al 1998). Furthermore, adult neurogenesis was reported to be associated with discrimination, learning and memory (Deng et al 2009, Gheusi et al 2000, Rochefort et al 2002). Therefore, it is essential to identify proper therapeutic targets for AD treatments without interfering with the normal function of APP.

The in vitro NSPC culture studies reported in chapter 3 and 5 indicated that APP may

participate in the regulation of NSPC proliferation and differentiation. In the study presented here, increased NSPC proliferation was observed in NSPCs derived from APP transgenic (Tg2576) mice while decreased NSPC proliferation was observed in cultures from APP KO mice (Chapter 3). However, enhanced NSPC proliferation was affected neither by the secreted soluble form of APP, sAPPα, nor by the β-amyloid protein, Aβ (Chapter 3). Instead, these studies demonstrated that APP-induced NSPC proliferation is mediated by secretion of the cysteine protease inhibitor, CysC. CysC was identified in the NSPC conditioned medium, and the secreted CysC also was found to also stimulate NSPC proliferation in vitro (Taupin et al 2000, Tavera et al 1992). This finding was supported by the observation that recombinant CysC could promote NSPC proliferation.

CysC is secreted and targeted extracellularly through the secretory system. APP overexpression has been reported to enhance vesicle exocytosis in PC12 cells (Lee et al 2008a). Thus, increased CysC secretion might be due to increased vesicle exocytosis of CysC into the cell culture media. In support of this idea, the studies presented in Chapter 3 showed that lower intracellular levels of CysC were detected in cell lysates of Tg2576 NSPCs compared to the corresponding WT NSPCs. In addition, the studies found higher CysC secretion levels in conditioned medium from APP overexpressing (Tg2576) cells, while lower levels of secreted CysC were present in APP KO conditioned medium. Surprisingly, there was no increase in *CysC* mRNA levels in Tg2576 cells compared to the corresponding wild type control cells. This result may suggest the possibility that APP overexpression in Tg2576 cells reaches a saturation point, so increased APP expression does not further increase *CysC* mRNA transcription. The overexpression of APP may play little role in *CysC* transcription,

but it may enhance CysC secretion in Tg2576 cells. Moreover, studies based on real-time PCR indicated that there were lower *CysC* mRNA levels in APP KO NSPCs compared to the corresponding wild type NSPCs (Chapter 3). This suggests that lower expression levels of APP, such as those that occur endogenously, may be able to regulate CysC transcription.

In agreement with this study, which showed increased CysC in APP NSPCs, APP overexpression has previously been reported to be associated with increased CysC levels in APP transgenic mice (Steinhoff et al 2001). In addition, CysC was found to colocalize with Aβ in vascular amyloid and in amyloid plaque cores of patients with AD and other neurodegenerative diseases (Haan et al 1994, Levy et al 2001, Maruyama et al 1990). Co-deposition of CysC and amyloid plaques (Fig. 6.1) was also observed in non-demented aged individuals (Levy et al 2001). Moreover, enhanced amyloid plaque degradation occurs in APP transgenic mice with CysC genetically deleted (Sun et al 2008), despite CysC having been shown to suppress amyloid deposition in AD mouse models (Kaeser et al 2007, Mi et al 2007). However, CysC deletion in human APP transgenic mice was reported to significantly lower Aβ in another study (Wang et al 2012). Therefore, a complex interplay between APP expression, Aβ plaque, CysC production and amyloid deposition may occur in AD. This needs further examination in future studies.

CysC null mice exhibited a decreased capability for neurogenesis (Pirttila et al 2005), which supports a role for CysC in NSPC proliferation. Thus, this suggests the possibility that manipulation of CysC expression or secretion may facilitate NSPC proliferation and thereby possibly enlarge the pool of stem cell treatments. Besides,

CysC is an endogenous inhibitor of cysteine proteases and it may regulate NSPC proliferation through inhibition of a protease. Indeed, some proteases have also been found to be associated with NSPC proliferation and differentiation (de Azevedo-Pereira et al 2011, Egberts et al 2004, Ekdahl et al 2001, Joyce et al 2004, Santos et al 2012, Watkinson 1999, Yadaiah et al 2013a). However, further studies of the mechanism by which cysteine protease activity is involved in NSPC proliferation or differentiation is required, and this may provide a new insights and understanding of stem cell biology.

The specific cysteine protease inhibitor, E-64, was also found to promote NSPC proliferation (Chapter 4). This result supports the idea that CysC may act as a protease inhibitor to stimulate cell growth, although CysC affinity chromatography did not purify any cysteine proteases that bind to CysC. E-64 is a non-cell membrane permeable cysteine protease, thus it may inhibit an extracellular cysteine protease to stimulate NSPC proliferation (Chapter 4). CysC was shown to inhibit several extracellular cathepsins, including cathepsins B, H, K, L, and S (Bernstein et al 1996), but it was inactivated by cathepsin D (Abrahamson et al 1991, Lenarcic et al 1991). Some cathepsins have been reported to induce neuronal apoptosis, therefore inhibition of these cathepsins may increase cell survival or perhaps cell growth (Boland & Campbell 2004, Kingham & Pocock 2001).

An imbalance between production and metabolism of cysteine proteases and their endogenous inhibitor may be involved in amyloid pathology in AD (Nakamura et al 1991, Sun et al 2008). Cathepsin B has been reported to effectively reduce Aβ, because inhibition of cathepsin B in mice overexpressing APP results in increased

plaque load (Mueller-Steiner et al 2006, Sun et al 2008, Wang et al 2012). In addition co-localization of cathepsins and senile plaques in the brain of AD patients has been reported (Cataldo & Nixon 1990). Furthermore, cathepsins B and L have also been implicated in APP processing (Klein et al 2009). These studies reveal a correlation between cysteine proteases and amyloid deposition. Therefore, further investigation into CysC or E-64 bound by which cysteine protease inhibitors (Chapter 4) may not only contribute to direct NSPC proliferation for stem cell therapy, but also contribute to modulate Aβ degradation for AD treatment.

APP also promoted enhanced neuronal and astrocytic differentiation of NSPCs. However, this effect was not caused by CysC, Aβ or sAPPα (Chapter 5). This result is consistent with another study from our group showing that APP overexpression can promote neuronal differentiation, and that APP-induced neuronal differentiation is regulated by the proneural basic helix-loop-helix (bHLH) transcription factor, neurogenin 2 (Ngn2) (Bolos et al 2014). Ngn2 plays an important role in driving neuronal differentiation during neurogenesis (Ali et al 2011, Nieto et al 2001), and Ngn2 alone is sufficient to improve differentiation of embryonic stem cells into mature neurons (Thoma et al 2012). Thus, this suggests that APP-induced neuronal differentiation of NSPCs is via modulation of Ngn2 expression (Bolos et al 2014). However, the basis of how APP activates Ngn2 transcription is still unclear.

As the extracellular fragments of APP-Aβ and sAPPα were not found to promote NSPC proliferation, this suggests that the intracellular domain of APP (AICD) liberated by γ - secretase cleavage of APP processing is involved in APP induced CysC gene expression (Fig 6.1). AICD has been reported to interact with nuclear

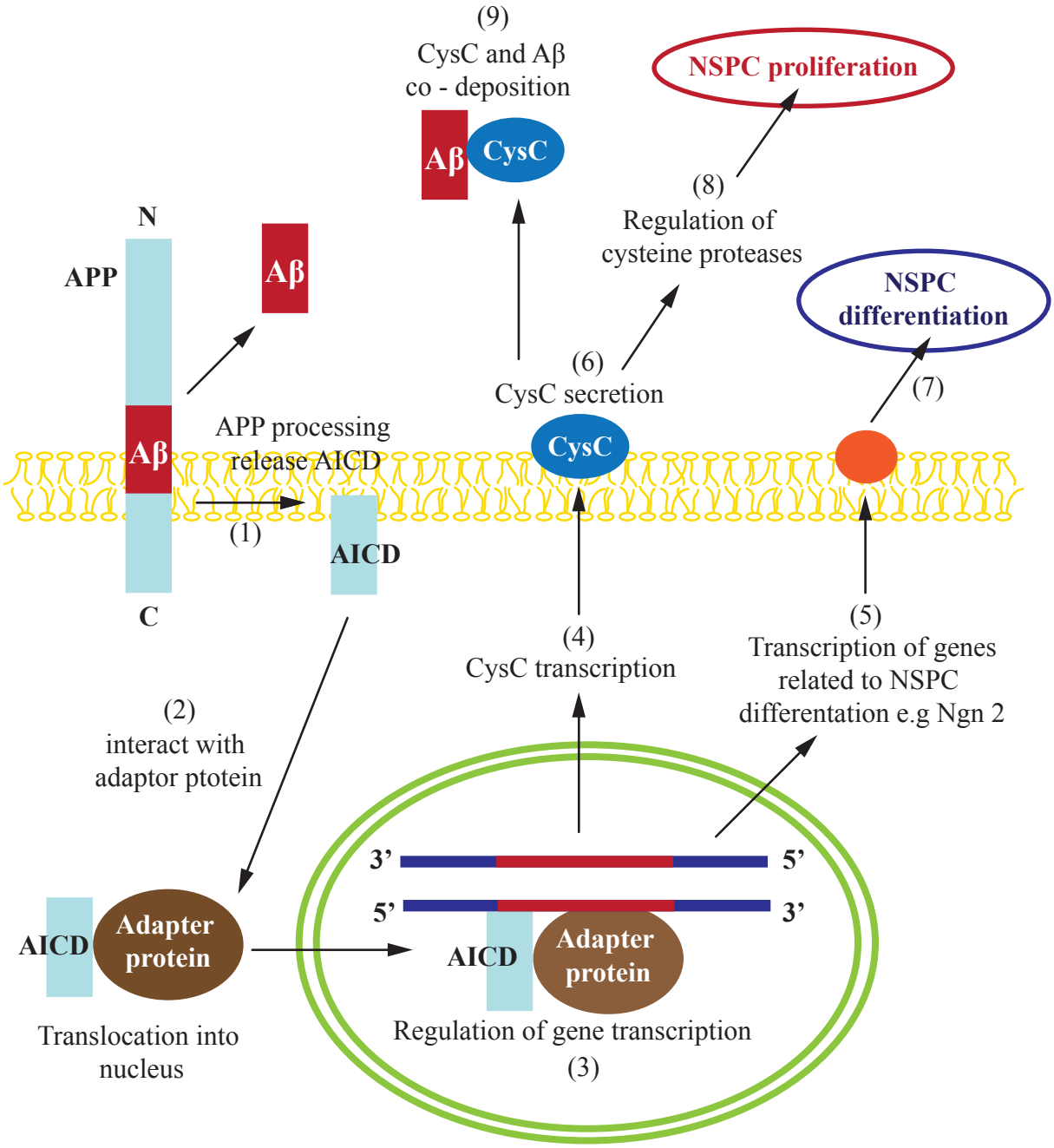

Figure 6.1 Model of the possible role of APP in NSPC proliferation and differentiation. Briefly, the APP intracellular domian (AICD) is released by APP processing (1) and then interacts with an adaptor protein to form a complex (2). The AICD- adaptor protein complex translocates into the nucleus and possibly regulates gene transcription (3) such that of *CysC* (4) or genes that relate to NSPC differentiation e.g Ngn2 (5). Secreted CysC (6) may either modulate cysteine protease activity to promote NSPC proliferation (8) or co-deposit with Aβ (9). AICD may also facilitate NSPC differentiation by activating the transcription of genes related to NSPC differentiation (7).

adaptor proteins to form complexes that translocate into the nucleus, where they regulate gene expression (Cao & Sudhof 2001, Cao & Sudhof 2004, Dawkins & Small 2014, Giliberto et al 2008, Kimberly et al 2005, von Rotz et al 2004, Xu et al 2007). However, AICD may negatively affect NSPC proliferation, as decreased NSPC proliferation has been observed in APP KO mice expressing AICD (Ghosal et al 2010). Nevertheless, NSPC proliferation remained normal at 6 weeks of age but was reduced at 3 months in APP KO mice expressing AICD (Ghosal et al 2010). Neuro inflammation has been suggested as a possible explanation for the effect of AICD on NSPC proliferation, as AICD has been reported to impair NSPC proliferation via inducing neuroinflammation (Ghosal et al 2010). The involvement of inflammation is supported by the finding that anti-inflammatiory agents can rescue defective NSPC proliferation (Ghosal et al 2010). Thus, the decreased NSPC proliferation in AICD transgenic mice may be due to neuroinflammation (Ghosal et al 2009, Ryan & Pimplikar 2005, Vogt et al 2011). The impaired NSPC proliferation in APP null mice expressing AICD may be due to activation of glycogen synthase kinase-3β (GSK-3β), a protein involved in cell apoptosis (Ghosal et al 2010). However, GSK-3β mRNA and protein levels were not altered in these AICD transgenic mice (Ghosal et al 2010, Hetman et al 2000, Ryan & Pimplikar 2005). Therefore, the mechanism of the effect on NSPC proliferation is unclear (Ghosal et al 2010). It is possible that CysC changes the cleavage of endogenous APP to AICD, which further affects NSPC proliferation and differentiation. Nonetheless, the current study provides evidence that CysC is downstream of APP (Chapter 3). However, whether AICD plays a role in modulation of CysC transcription is not yet confirmed and will need further study.

In addition, the extracellular domain fragments of APP, namely Aβ and sAPPα, were not found to promote neuronal or astrocytic differentiation in the present study. Thus this supports the idea that the intracellular domain of APP may be involved in mediating this effect, via activation of gene transcription (Fig 6.1). AICD may be a transcription regulating factor (Cao & Sudhof 2001, Cao & Sudhof 2004, Giliberto et al 2008, Kimberly et al 2005, von Rotz et al 2004, Xu et al 2007). For example, AICD has been reported to facilitate the nerve growth factor response in neuronal cell differentiation and promote neurite outgrowth as well as to improve axonal elongation and path finding (Ryan & Pimplikar 2005, Zhou et al 2012). Nevertheless, other studies indicate that AICD suppresses neuronal specification of NSPCs or induces neuronal specific apoptosis (Ohkawara et al 2011, Shu et al 2015). However, APP KO mice that overexpress AICD exhibited normal neuronal differentiation of NSPCs (Ghosal et al 2010). Therefore, whether AICD functions as a gene transcriptional factor to regulate expression of genes controlling NSPC differentiation needs further investigation.

In summary, the main finding of this study was that APP overexpression induced NSPC proliferation and differentiation (Chapter 3 and 5), and that APP may plays a stimulatory role in adult NSPC neurogenesis. For this reason, AD therapies based on altering APP processing may have to be considered carefully in order to not interfere with any normal physiology related to APP function in the human brain. In addition, APP-induced neuronal differentiation may be mediated via *Ngn2* transcription (Bolos et al 2014), whereas the APP induced NSPC proliferation was found to be due to secretion of the cysteine protease inhibitor CysC. It is possible that CysC is released from the cells by non – secretory mechanism (i.e following cell death). However,

previous studies have shown that CysC is actively secreted (Taupin et al 2000). Thus, the presence of CysC in the culture medium is likely to be due to active secretion. This idea is further supported by the observation that most of the CysC was in the culture medium rather than in the cell lysate fraction. Furthermore, low concentrations of other cysteine protease inhibitors such as E-64 and antipain also stimulated NSPC proliferation in this study. As modulation of cysteine proteases activity is suggested to be related to amyloid pathology in AD (Klein et al 2009, Sun et al 2008, Wang et al 2012), further investigations of the molecular basis of APP's involvement with NSPC proliferation and differentiation, and the role of CysC, may not only contribute to the development of new stem therapies for neurological diseases such as AD, but may also provide an understanding of the underlying causes of neurodegeneration.

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